

DEPARTMENT OF CLINICAL NEUROSCIENCE  
Karolinska Institutet, Stockholm, Sweden

# **NEUROINFLAMMATORY BIOMARKERS IN SUICIDAL BEHAVIOR**

Josef Isung



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# NEUROINFLAMMATORY BIOMARKERS IN SUICIDAL BEHAVIOR

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Josef Isung**

*Principal Supervisor:*

Associate Professor Jussi Jokinen  
Karolinska Institutet  
Department of Clinical Neuroscience  
Division of Psychiatry  
Professor  
Umeå University  
Department of Clinical Science  
Division of Psychiatry

*Co-supervisor(s):*

Professor Fredrik Piehl  
Karolinska Institutet  
Department of Clinical Neuroscience  
Division of Neuroimmunology

Professor Bo Runeson  
Karolinska Institutet  
Department of Clinical Neuroscience  
Division of Psychiatry

Associate Professor Peter Nordström  
Karolinska Institutet  
Department of Clinical Neuroscience  
Division of Psychiatry

*Opponent:*

Professor Yogesh Dwivedi  
UAB School of Medicine  
Department of Psychiatry  
Division of Behavioral Neurobiology

*Examination Board:*

Professor Christina Dalman  
Karolinska Institutet  
Department of Public Health Sciences  
Division of Public Health Epidemiology

Associate Professor Mussie Msghina  
Karolinska Institutet  
Department of Clinical Neuroscience  
Division of Psychiatry

Associate Professor Fotis Papadopoulos  
Uppsala University  
Department of Neuroscience  
Division of Psychiatry



*'All truly great thoughts are conceived while walking.'*

Friedrich Nietzsche, *Twilight of the Idols*

*To Johanna, my love*

## ABSTRACT

Immune dysregulation is of importance in the pathophysiology underlying psychiatric disorders including major depression, bipolar disorder, and schizophrenia. Neurobiological alterations such as a dysregulated stress-hormonal axis and serotonergic alterations have been reported in suicide attempters at risk for subsequent suicide. Immune dysregulation in suicidal behavior is less well studied, but with some evidence for elevated proinflammatory cytokines and decreased levels of neurotrophins, such as brain-derived neurotrophic factor. The aim of this thesis was to study neuroinflammatory biomarkers with regard to suicidal behavior in patients with mood disorders.

For the first part of this thesis (**Studies I-III**) we studied a high-risk cohort of patients with a recent suicide attempt. All were thoroughly assessed diagnostically and rated with regard to personality traits, severity of depression and suicidal intent. We did a follow-up on all patients regarding cause of death. 20 healthy controls were used for comparison. Patients were sampled for blood (n = 58) and cerebrospinal fluid (CSF) (n = 43). Samples were assayed using immune-based assay-systems for immune markers and growth factors.

Seven patients who at follow-up, had completed suicide had lower plasma levels of vascular endothelial growth factor (VEGF). Furthermore, we found that the patients had lower CSF levels of both VEGF and interleukin- (IL-) 8 compared to the healthy comparison group. Lastly, we found that IL-6 levels in both plasma and CSF correlated with personality traits of impulsivity in suicide attempters.

For the second part of this thesis (**Study IV**) we investigated the effects of physical activity on immune markers implied to be of importance in the pathophysiology of depression and suicidal behavior. The participants were completely healthy both somatically and psychiatrically. We studied the effects of an acute intensive exercise challenge during four days (n = 14), and a moderate exercise challenge during four weeks (n = 13). Paired sampling from blood and CSF before and after exercise intervention was compared. All rated mood before and after intervention.

We found that CSF IL-8 was significantly elevated in both groups as a result of physical exercise. Serum IL-6 and IL-8 were significantly elevated in the total group, as well as IL-6 in the intensive group and IL-8 in the moderate group. We found no significant correlation between serum and CSF levels in the assayed analytes, suggesting that the effects of physical activity were segregated between the compartments. Furthermore, we found a negative correlation regarding mood ratings and CSF IL-8, suggesting relevance of CSF IL-8 as a state marker for mood.

**We propose that low VEGF may be a marker for treatment resistance and suicide risk, while high IL-6 seems to be related to impulsivity and violent methods of attempted suicide, both of which are important endophenotypes of suicidal behavior. Physical exercise is also an important confounder in immune biomarker studies.**

## LIST OF PUBLICATIONS

- I. **Isung, J.**, F. Mobarrez, P. Nordström, M. Asberg, and J. Jokinen. “Low Plasma Vascular Endothelial Growth Factor (VEGF) Associated with Completed Suicide.” *The World Journal of Biological Psychiatry: The Official Journal of the World Federation of Societies of Biological Psychiatry* 13, no. 6 (September 2012): 468–73. doi:10.3109/15622975.2011.624549.
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- III. **Isung, J.**, S. Aeinehband, F. Mobarrez, P. Nordström, B. Runeson, M. Asberg, F. Piehl, and J. Jokinen. “High Interleukin-6 and Impulsivity: Determining the Role of Endophenotypes in Attempted Suicide.” *Translational Psychiatry* 4 (2014): e470. doi:10.1038/tp.2014.113.
- IV. **Isung, J.**, M. Granqvist, J. Jokinen, and F. Piehl. “Immune Marker Variability in Plasma and Cerebrospinal Fluid after Physical Exercise: Implications for Biomarker Research in Psychiatry.” Manuscript.



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## LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (Serotonin)
5-HTTLPR	Serotonin transporter-linked polymorphic region
ACTH	Adrenocorticotrophic hormone
BDNF	Brain derived neurotrophic factor
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELA	Early-life adversity
GWAS	Genome-wide association studies
HPA	Hypothalamic-pituitary-adrenal
IDO	Indoleamine 2,3-deoxygenase
IFN- $\gamma$	Interferon- $\gamma$
KSP	Karolinska Scale of Personality
MADRS	Montgomery Åsberg Depression Rating Scale
MDD	Major depressive disorder
PE	Physical exercise
PHQ-9	Patient Health Questionnaire 9
PNS	Parasympathetic nervous system
PTSD	Post traumatic stress disorder
RDoC	Research Domain Criteria
SIS	Suicide Intent Scale
SNS	Sympathetic nervous system
SRI	Serotonin reuptake inhibitor
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
VEGF	Vascular endothelial growth factor

# 1 INTRODUCTION

## 1.1 SUICIDE AND SUICIDAL BEHAVIOR

*‘O happy dagger!*

*This is thy sheath; there rust, and let me die.’*

William Shakespeare, Romeo and Juliet, act 5, scene 3, 169-170.

Suicidal behavior is as old as mankind. In literature the tragedy surrounding suicide is often depicted in a romantic and melodramatic way. The issue of suicide has constantly been subjected to philosophical and ethical considerations where religious and societal credos have heavily influenced our own views and ideas. Suicidal behavior is common and most people have in one way or another been affected by it. Therefore taking an interest in this topic and finding ways to better understand and prevent it is quite an obvious task. This has been my rationale when undertaking this scientific project, being a clinician working daily with psychiatric disorders and being interested more generally in human behavior.

### 1.1.1 Scope

Suicide and suicidal behavior constitutes a major health concern worldwide which leads not only to severe morbidity or premature death, but also to suffering and disability for the surrounding family and friends. To prevent suicide is a top priority for the clinician, but suicide risk assessment also constitutes one of the most complex psychiatric assessments (1–3). More objective measures are urgently needed.

### 1.1.2 Suicide nomenclature

A consensus nomenclature for understanding and communicating suicide and suicidal behavior in research separates suicide and suicidal behavior in three main groups (4).

1. Completed suicide (CS). Death from injury that was self-inflicted and convincing evidence that the victim intended to kill himself/herself.
2. Suicide attempt (SA). Injurious or non-injurious where the attempt was self-inflicted and there is convincing evidence that the victim intended to kill himself/herself.
3. Suicide ideation (SI). Expressed thoughts of wanting to engage in suicidal behavior.

Suicide attempts are further classified as violent or nonviolent based on epidemiological findings that the method of attempt has important clinical implications and is predictive of a risk of future attempts and completed suicide (5,6). Self-poisoning is considered to be a nonviolent attempt. All others (e.g., shooting, hanging, attempted drowning or gassing) are considered to be violent.

### 1.1.3 Epidemiology

The global report on suicides from 2012 estimated the number of individuals for whom the cause of death is suicide to be more than 800,000. Suicide attempts are reported to be 10–20 times more frequent and, as opposed to completed suicide, to be more frequent among females (7). The global rate is about 11.4 per 100,000 individuals (15.0 for males and 8.0 for females) (7). In the 15–29 age group, suicide is the second leading cause of death globally. In Sweden, 1524 people committed suicide in 2014 (1042 males and 482 females), indicating a suicide rate of 19.0 per 100,000 individuals. In the 15–24 age group, suicide is the most common cause of death in Sweden for both males and females (8).

### 1.1.4 What causes suicidal behavior?

Mental disorders are the most prominent risk factor for suicide or suicidal behavior with up to 90% of all victims having definable psychiatric diagnosis (1,9). For common mental disorders such as depression, bipolar disorder, schizophrenia, borderline personality disorder, anorexia nervosa and some substance-use disorders, the risk exceeds 10 times that of the general population (10). Major depressive episodes as part of either a major depressive or bipolar disorder account for at least half of suicides (9). However, suicide and suicidal behavior are highly complex and multi etiological and far from all patients with mental disorders commit suicide.

## 1.2 RISK FACTORS FOR SUICIDAL BEHAVIOR

Identified risk factors underlying suicidal behavior can be divided into so called proximal and distal factors, where proximal factors are precipitating events such as acute crisis or depressive disorder known to be a direct driving force towards suicidal behavior, and distal factors are underlying risk factors that predisposes any individual to suicidal behavior including early life adversity and heritable factors, also sometimes described in the stress-diathesis model for psychiatric disorders (9,11–13).

### 1.2.1 Genetics

Suicidal behavior runs in families, and twin-studies have estimated the heritability to be around 30-50% (9). However, suicidal behavior is complex and, on adjusting for other psychological conditions, the heritability estimate for suicide attempt is 17.4% and for suicidal ideation 36% (9). No specific gene linked to suicidal behavior has yet been identified, and genome-wide association studies (GWAS) have provided inconclusive results. Disorders generally described as having a complex underlying pathophysiology are frequently inconclusive in GWAS attempts, and it is not surprising that, so far, we have not seen more robust findings. Psychiatric genetics is however on the rise, with increasing sample sizes being assembled through collaborative research efforts such as the Psychiatric GWAS Consortium, and also with the help of better and increasingly less expensive technology doing, e.g., whole genome sequencing, where recent reports are encouraging (14–17).

### 1.2.2 Stress and the HPA axis

The main regulator of homeostasis is the stress response system, mainly consisting of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system, constituted by the sympathetic nervous system (SNS) and its counteracting part, the parasympathetic nervous system (PNS) (18). Homeostasis is achieved by a constant regulation of physiological and behavioral responses challenged by external or internal stimuli (stressors). The SNS is the swift response system excreting norepinephrine (NE) from the locus coeruleus (LC) in response to stressors, while the HPA axis is the slower, but long-term regulator of stress homeostasis.

The HPA axis, acts via hypothalamic excretion of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), which, in a synergistic manner, target the pituitary via the portal hypophyseal circulation (18). From the pituitary gland adrenocorticotrophic hormone (ACTH) is excreted reaching its target organ the adrenal glands that secrete glucocorticoids (GCs). GCs are ubiquitous molecules, with a pleiotropic effect on multiple targets regulating bodily functions and the brain in response to stressors. GCs target glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), which having different affinities, where GRs respond to higher concentrations of GC and MRs respond to lower concentrations, the MRs being important for the overall HPA axis tone. GC can cross the blood brain barrier and targets back at diverse brain regions, among others, the hypothalamus and pituitary, to resolve the stress response via a negative feedback loop mechanism (18).

There is an intricate cross-talk between the immune system and the stress response system (19). An immune challenge triggers an acute inflammatory response, with an increase in proinflammatory cytokine activity. GC is also released during a stressor, such as infection, contributing, however, to a shift from proinflammatory domination with T helper (Th)1 to Th2 activity, thereby contributing to inflammation resolution via secretion of anti-inflammatory cytokines such as IL-4 and IL-10. Stress hormones may, however, in some settings, contribute to an increased release of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and IL-18 or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (19). The cross-talk between the stress response system and the immune system may, in some settings, malfunction, leading to deleterious chronic inflammatory conditions, characterized by an aberrant proinflammatory state and lack of inflammation resolution, contributing to the wide array of conditions that are clustered within the metabolic syndrome, such as atherosclerosis, certain cancers, autoimmune disease, and obesity, as well as depression (19).

HPA axis dysregulation can be quantitatively assessed. The dexamethasone suppression test (DST) is a test in which cortisol is assessed the morning after the subject is provided with the glucocorticoid analogue, dexamethasone, given before bedtime. Displaying low levels of cortisol, suggests being a suppressor (hypoactive HPA axis) and high levels suggest being a nonsuppressor (hyperactive HPA axis). Melancholic depression is characterized by a hyperactive HPA axis whereas atypical depression seems to be more related to a hypoactive HPA axis tone (18). Severe stress-reactions that are capable of inducing post traumatic stress disorder (PTSD), change the stress response system with a shift to a more depleted state as seen regarding NE

production within the LC (20) and a change in feedback mechanisms within the HPA toward a more hypoactive state (21). Regarding suicidal behavior there are inconsistencies, whereby some have reported associations between a hyperactive HPA axis, indicated by the DST, and an increased risk of completed suicide (22–25); however, there are also studies indicating that a hypoactive HPA axis is related to suicidal behavior (26,27).

Hypothetically, these diverse links could be evidence of different phenotypes, characterized either by a blunted HPA axis that could be the result of repeated depressive episodes initially characterized by hyperactivity, but with long-term effect being a “worn-out” system with a more hypoactive HPA axis (26,27). The other phenotypical characteristic would be one with a more hyperactive HPA axis which also may be mediated by early-life adversity (ELA) (13), where constitutively high levels of cortisol contributes to symptoms of anxiety and may have debilitating effects on cognition.

### 1.2.3 Early-life adversity (ELA)

ELA has repeatedly been found to be a highly relevant risk factor underlying neuropsychiatric disorders, including suicidal behavior (28). ELA does not only impact the risk for neuropsychiatric disorders but also disorders related to the metabolic syndrome, such as diabetes or obesity (29). Disturbance of the HPA axis with HPA axis hyperactivity and effects on developmental processes such as personality traits may mediate the risks that follow ELA events (13).

The evidence base is robust from animal studies (both rodents and primates) and studies in humans where e.g., epigenetic regulation (hypermethylation) is seen in the promoter region of the cortisol receptor in subjects who have experienced ELA (9). Further epigenetic regulation of the serotonin transporter and neurotrophic factors, mainly brain-derived neurotrophic factor (BDNF) is evident from animal studies and a down-regulation of mRNA coding for BDNF is seen in post-mortem studies on suicide victims with a history of ELA (9,29).

### 1.2.4 The serotonergic system

The serotonergic system has been studied in the context of psychiatric disorders for decades. Initially, findings by, e.g., Åsberg and collaborators (30,31) provided evidence for altered serotonergic neurotransmission in suicide attempters where low levels of the main serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), was found in the CSF, and even though subsequent studies and a review questioned these results, a later meta-analysis could report low CSF 5-HIAA in attempters and also that low levels predicted future new attempts and completed suicide (32).

Further studies, mostly postmortem, have extended the evidence for an altered serotonin neurotransmission in the brain, raising hopes that candidate gene studies would provide genetic risk markers for suicidal behavior (33). However, unfortunately, candidate gene studies with a focus on different alleles of tryptophan hydroxylase,



monoamine oxidase A (MAOA), or the serotonin transporter-linked polymorphic region (5-HTTLPR) have provided inconsistent results (13).

### 1.2.5 Personality

The concept of endophenotypes includes stable personality traits, which are believed to be highly genetically determined and relatively stable throughout the course of life. There is a long research tradition connecting personality traits as predisposing factors underlying the risk of psychiatric disorders including suicidal behavior. The concept of trait neuroticism with a disposition to negative emotion and anxiety is a strong risk factor for depressive disorder also predictive of recurrence and course (34,35).

Regarding suicidal behavior, personality traits dominated by impulsivity and aggression are contextualized as important endophenotypes (36). The concept of impulsivity is a multifaceted construct, and the foremost domains that can be tested with neuropsychological test batteries are more robust and have a linkage to gene variants rather than results from personality inventories (37). These traits are the ones mostly replicated in studies on suicide risk, and seem to be especially important risk markers among younger subjects. Impulsivity and aggressiveness run in families, with evidence of strong heritability but an environmental influence is also found. The time-frames for environmental influence seem to be mostly prominent during susceptible developmental phases such as in childhood and/or adolescence (36,38–42).

There are population-based cohort studies that have assessed proinflammatory cytokines, mainly IL-6, and linked high levels to personality traits further associated with psychiatric and somatic morbidity (43–45). One of these studies could report an association between impulsivity measured with the NEO-PI-R (based on the five-factor model of personality) and higher levels of IL-6 (45) while another population-based study associated a more crude measure (white blood cell counts) and found an association with impulsivity (46). There are, however, associations between these traits and risk-taking habits such as smoking and between visceral fat accumulation and increased levels of proinflammatory cytokines (47), which could partly explain this link. Thus, impulsivity seems to correlate with both risk-taking habits and increased IL-6 levels in the periphery and these associations may explain why there is an increased risk for morbidity and mortality connected to the same cytokine aberrations (43,45,48,49).

When it comes to aggressive behavior, links have been reported between serotonergic regulation and, more recently, IL-6 and C-reactive protein (CRP) levels (37,50). A Swedish study has recently reported a connection between the acute-phase protein CRP and personality, with a genetic association. The authors reported an association between a *CRP* gene polymorphism with the personality domain Extraversion and its subscale Impulsiveness, measured with the Karolinska Scale of Personality (KSP) (51).

## 1.3 ENDOPHENOTYPES AND RDOC

As a consequence of disappointment in psychiatric research concerning translating promising results from preclinical studies into subsequent clinical studies, the core

concept of categorical psychiatric diagnostics has been called into question by the National Institute for Mental Health (NIMH). There take on this has been the so-called Research Domain Criteria (RDoC), emphasizing endophenotypic traits or biological substrates as more stable and valid constructs to bridge the gap from bench to bedside since diagnostic heterogeneity has long been appreciated and thought of as an important obstacle in research (52).

The RDoC structure is completely agnostic regarding the Diagnostic and Statistical Manual of Mental Disorders (DSM) system, and the RDoC system is an ongoing framework that is constantly fueled by new findings in the field of biological psychiatry (52). The concept is also based on psychiatric disorders, including personality disorders, as being dimensional, rather than categorical. Since NIMH has promoted an emphasis on RDoC in research, there have been recent discussions about and criticism of both viewpoints, so perhaps, at the moment, keeping both perspectives may be the most relevant take while waiting for further knowledge to emerge from research (53–55).

## **1.4 BIOMARKERS IN PSYCHIATRY**

### **1.4.1 What do we mean by biomarkers?**

Biomarkers are biological variables that can have different features depending on what the biomarker is intended to describe.

Davis and collaborators (56) propose a division of biomarkers into six different features;

1. Biomarkers of risk, intended to predict an individual's risk of developing any disorder;
2. Biomarkers of diagnosis/trait, intended to be of help in the diagnostic process;
3. Biomarkers of state/acuity, intended to measure the severity of the disorder;
4. Biomarkers of stage, intended to reflect and measure staging in any disorder;
5. Biomarkers of treatment response, intended to be of value for the clinician in predicting treatment response and individualized treatment;
6. Biomarkers of prognosis, intended to predict the outcome for any individual (56).

Furthermore, Pine and Leibenluft (57) argue that when thinking of biomarker research in psychiatry one cannot be satisfied with biomarkers that are simply predictive of treatment response and prognosis, but an intensified effort is needed to search for biomarkers closer to a mechanistic focus to align with research that has been instrumental to the great success that has been the case in other fields of medical research (57). While psychiatric research is inherently destined to be more complicated than, e.g., the same efforts in cardiology or infectious disease due to the complexity of the brain, a shift in focus on biomarkers that are narrow to mechanisms would be of great value for bringing leverage to the understanding and treatment of psychiatric disorders (57).

## **1.5 NEUROINFLAMMATION HYPOTHESIS OF DEPRESSION**

The current concept of the neuroinflammation hypothesis of depression was initially proposed by Smith in 1991, arguing for a role of macrophages in the pathophysiology of depression (58). However, even earlier, the role of infectious diseases and the immune system has always been a recurrent topic as in, e.g., the “focal infection theory” that had its highlight during the twenties and thirties as one overarching theory which argued that many systemic diseases, including schizophrenia and bipolar disease, had their origin in focal infections, primarily of dental origin (59).

The neuroinflammation hypothesis postulates, based on the recognition of symptom overlap seen during states of infection (sickness behavior) and depression, such as depressed mood, fatigue, problems with concentration, lack of appetite, and insomnia, that an immune system dysregulation may be a pathophysiological mechanism underlying some cases of clinical depression (60–62).

Based on this hypothesis, an increasing amount of preclinical and clinical research has focused on finding evidence that immune markers are involved and dysregulated, and indeed accumulating evidence has shown increased concentrations of proinflammatory cytokines (such as IL-6 and TNF- $\alpha$ ) in major depressive disorder (MDD) and, more recently, in suicidal behavior (63–66).

Corroborative evidence for the neuroinflammation hypothesis has also come from the clinical treatment that has been used for years for hepatitis C, in the form of interferon- $\alpha$ , a powerful inducer of IL-6. This treatment has proved to be an experimental model for the induction of clinical depression, which is not distinguishable from a major depression, that is induced in up to 50% of treated patients, including the risk of suicidal behavior in some individuals (60,67,68). There is further evidence from post mortem studies reporting signs of increased mRNA levels of proinflammatory cytokines in the orbitofrontal cortex of suicide victims, as well as signs of microgliosis in suicide victims (69–71).

## **1.6 NEUROTROPHIC HYPOTHESIS OF DEPRESSION**

The neurotrophic hypothesis of depression suggests that the pathophysiological pathway to depressive state also involves neurotrophic factors. Mainly, the idea is that neurotrophins important for cell survival, growth, maturation, synaptogenesis etc. (implying that they have an adverse effect on neuroplasticity) are affected and down-regulated in response to, e.g., long-term stress, ELA via epigenetic regulation, acute stress or other genetic or environmental contributions to neurotrophin dysfunction (72–74).

BDNF has been most extensively studied to this end, but also VEGF, nerve growth factor (NGF) and fibroblast growth factor (FGF) are targeted in these investigations (75–77). Studies have proposed mechanisms for how induction of depressive states can be understood via cytokine activation of different pathways targeting the brain and affecting the HPA-axis, monoaminergic systems and growth factor expression

(72,74,78,79). Indeed, exercise can also affect neurotrophins with evidence of increased BDNF and VEGF (80,81)

## **1.7 EXERCISE PHYSIOLOGY**

Physical activity is key in humans for maintaining health, homeostasis and preventing disease. Physical exercise (PE) is defined by the American College of Sports Medicine (ACSM), as “planned, structured and repetitive bodily movement done to improve or maintain one or more components of physical fitness” (82). There are several studies that report the effects of PE on different components of the immune system, where, e.g., the cytokine IL-6 is known to be released directly from the muscle fibers, and the systemic levels can increase 100-fold after a single prolonged session of physical exercise (83).

The acute effects of PE are analogous to an acute stress-like reaction, with direct effects on the HPA axis and the SNS, resulting in the secretion of cortisol and epinephrine (83). The beneficial health effects of physical exercise are probably attributable to the long-term anti-inflammatory effects promoted by regular physical activity.

Cortisol and epinephrine inhibit the release of TNF- $\alpha$  secreted by monocytes. IL-6 also contributes to this down regulation and even adds to a further secretion of cortisol. IL-6 also stimulates the release of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA) from monocytes. PE also mobilizes regulatory T-cells ( $T_{Reg}$ ) a major source of another anti-inflammatory cytokine, IL-10. Chemokine release in the periphery is also a known effect, where repeated increases in chemokines due to PE leads to a down-regulation of chemokine receptors and, as an effect of this, reduced tissue infiltration in the long term (83–85).

## **1.8 THE ROLE OF CYTOKINES IN SUICIDAL BEHAVIOR**

The number of papers reporting on differences in cytokines related to suicidal behavior is increasing every year. Notwithstanding the increasing number, a certain amount of disappointment has arisen within the field of psychoneuroimmunology since the individual study results are difficult to replicate and inconsistencies are also common. Quite a few reviews and even meta-analyses have been published just during the last few years, mirroring the number of reports that have reached peer-reviewed journals (86–89).

### **1.8.1 Cytokines in suicidal behavior**

There are several studies that have examined cytokines in relation to suicidal behavior. Mostly proinflammatory cytokines have been investigated where increased IL-1, IL-6, and TNF- $\alpha$  are reported to be the most consistent findings (86). However, low IL-2 and increased interferon- $\gamma$  (IFN- $\gamma$ ) levels have also been reported (87–89). IL-6 is arguably the most promising biomarker, since IL-6 increase is reported not only in the periphery but also in the CSF and in some studies even distinguishing suicide attempters with depression, non-attempters with depression, and healthy controls (63,65).

For understanding of the mechanistic consequence of aberrant cytokine activity, an interesting connection with the kynurenine pathway is highlighted (90,91). Inflammation knowingly induces this pathway, where proinflammatory cytokines activate the rate-limiting enzyme indoleamine 2,3-deoxygenase (IDO) inducing tryptophan catabolism to kynurenine and reducing the production of serotonin (5-HT) (see Fig. 1) (74,92). The main catabolites in this pathway, kynurenic acid (KYNA) and quinolinic acid (QA), both have diverse effects directly modulating the main excitatory neurotransmitter system glutamate, which has also been directly associated with suicidal behavior in one study (74,90,92).

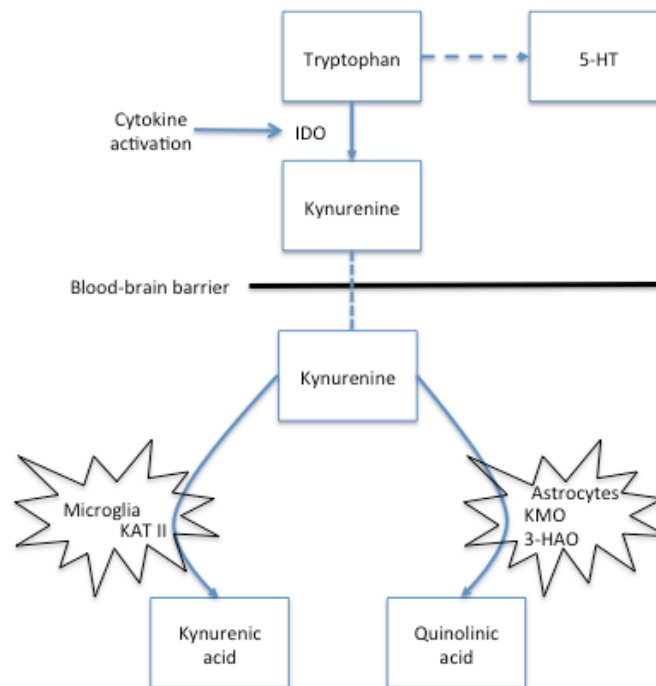


Figure 1. **Activation of the kynurenine pathway.** Cytokines can induce the kynurenine pathway in inflammation conditions in both the periphery and the CNS. Pathway activation via IDO leads to tryptophan depletion (precursor also to 5-HT). In the periphery macrophages and dendritic cells can produce kynurenine, and in the central nervous system (CNS) microglia and astrocytes are involved in the farther downstream catabolism to CNS active KYNA and QA via KAT II or KMO and 3-HAO. KYNA can reduce glutamate release being an antagonist, and QA can increase glutamate release via NMDA-R activation, thus contributing to CNS excitotoxicity and eventually neurodegeneration. (Adapted from Haroon et al.) (74).

## 1.9 THE ROLE OF NEUROTROPHINS IN DEPRESSION AND SUICIDAL BEHAVIOR

### 1.9.1 Brain-derived neurotrophic factor (BDNF)

BDNF, probably the most important neurotrophic factor, is also the most extensively studied marker in relation to depression and suicidal behavior. An exhaustive review concerning its role provides compelling evidence since peripheral BDNF levels are generally lower in suicide attempters, distinguishing even nonsuicidal depressed individuals, and low expression levels and mRNA levels are reported within the

prefrontal cortex and hippocampus in post-mortem studies, distinguishing suicide completers regardless of the psychiatric diagnosis (93). BDNF is also clearly regulated and affected by the HPA axis and stress and, in that respect, a further link with suicidal behavior could be contextualized (93).

### 1.9.2 Vascular endothelial growth factor (VEGF)

VEGF belongs to a family of proteins with different sources of origin and target effects (94). It was named VEGF since it was originally recognized as a key angiogenic protein vital for embryonic survival (95). VEGF-A, the prototypical member, has several isoforms in which two of the splice variants are predominant in the brain (94). There are also known single nucleotide polymorphisms which, in one study, have been shown to correlate with hippocampus morphology (96). The effect of VEGF is exerted mainly via the VEGF receptor-1 (VEGFR-1) and 2, VEGFR-2 being the most extensively studied (97). There is increasing evidence for VEGF to be neuroprotective and to have neurotrophic effects (94,98). Based further on studies of neuroprotective aspects, VEGF has also been suggested to be important in the pathophysiology of neurodegenerative disorders such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), as well as Alzheimer's disease (94,97). VEGF is also implicated in stress-related disorders and depression (73); however, prior to our reports, it has not been studied in relation to suicidal behavior.

## 2 AIMS

The general aim of this thesis was to study whether neuroinflammatory biomarkers could be predictive of suicidal behavior and/or completed suicide. The first part of the thesis (studies I-III) was based on a well-characterized clinical sample of suicide attempters with a long-term follow-up in the Swedish national registers. The second part of the thesis (study IV) was based on a well-characterized sample of healthy participants without any history of psychiatric or somatic morbidity.

**Study I:** A follow-up study of suicide attempters in which immune markers, mostly proinflammatory cytokines and growth factors, were assessed concerning their predictive value for completed suicide with the aim of identifying new inflammatory biomarkers for suicide prediction.

**Study II:** A cross-sectional study in which we compare levels of proinflammatory cytokines and vascular endothelial growth factor in the CSF in suicide attempters and healthy controls.

**Study III:** A cross-sectional study in which we assess the relationship between CSF and plasma interleukin-6, personality traits, and the method of attempted suicide in suicide attempters.

**Study IV:** A longitudinal study on the effects on and variability of immunological biomarkers in healthy participants after two different physical exercise paradigms involving blood and CSF.

## 3 METHODS

### 3.1 INCLUSION PROCEDURE

#### 3.1.1 Subjects, Studies I-III

##### 3.1.1.1 Study I

For our first study, patients with a suicide attempt and having their clinical follow-up at the Psychiatric Clinic at the Karolinska University Hospital were asked to participate in a study on biological and psychological risk factors for suicidal behavior. The study protocol was reviewed and approved by the Regional Ethical Review Board in Stockholm (Dnr 93-211). All participants gave their written informed consent for the study.

The study design is a follow-up study on a cohort of 58 suicide attempters (23 men; mean age, 39 years; SD = 12.7; range, 20–69 and 35 women; mean age, 36 years, SD = 12; range, 18–68). Patient recruitment was carried out during the years 1993–1998.

Inclusion criteria were as follows:

- Age of 18 years or older
- A recent suicide attempt (within a time limit of one month)
- An adequate capacity to communicate verbally and in writing in the Swedish language.

Exclusion criteria were as follows:

- Psychotic disorder
- Dementia
- Mental retardation
- Intravenous drug abuse

Suicide attempt was defined as any nonfatal, self-injurious behavior with the intent to die. Eleven of the recruited patients (19%) had used a violent method when attempting suicide (6).

The patients included were interviewed by a trained clinical psychiatrist using the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (SCID) (99) I, Research Version, when assessing the diagnosis in accord with the, at the time, current version of the DSM-III (99). The SCID II interview was used to assess the occurrence of axis II diagnoses.

For the full cohort included in this study, the following diagnostic data were confirmed: 93% had at least one current axis I psychiatric diagnosis; 78% of the patients satisfied the criteria for some mood disorder, 7% for an adjustment disorder, and 3% for any anxiety disorder. One patient had a substance-use disorder and one an unspecified psychiatric disorder (not psychotic). A comorbid substance-use disorder was found in 24% of the cohort, where the majority had an alcohol-use



disorder. Patients that satisfied the criteria for some personality disorder amounted to 39% of the cohort. Forty-seven patients (81%) were drug-free; 7 patients had been on antidepressant medication, mostly SRI.

Table 1. Demographic and clinical data concerning the study population, survivors vs. victims.

Variable	Surviving suicide attempters (n = 51)	Suicide victims (n = 7)
Age, mean $\pm$ SD	37 $\pm$ 12.3	37 $\pm$ 12.6
Gender, male/female	19/32	4/3
BMI, mean $\pm$ SD	24.3 $\pm$ 3.9	26.3 $\pm$ 3.1
MADRS, median (IQR)	17(10–23)	12.1(3–21)
SIS, median (IQR)	15.7(11–21)	20.1(18–22)

To determine outcome, defined as completed suicide on follow-up, we used the unique Swedish personal identification number and linked it to the National Swedish Cause of Death Register, regulated and controlled by the National Board of Health and Welfare (<http://www.socialstyrelsen.se/register/dodsorsaksregistret>). Before January 2009, seven of the original 58 patients had committed suicide. Suicides were ascertained from death certificates. Five of the patients had committed suicide within 6 years and two after 11 years from originally entering the study.

Table 2. Descriptive statistics on the outcome measure, completed suicide.

Variable	Suicide completers (n = 7)
Time to suicide (median, mean, range)	4 years, 6 years, 1.7–12.8 years
Follow-up time (range)	11–15 years

### 3.1.1.2 Studies II and III

Patients included in Studies II and III were the same as those described in Study I with the exemption that only 43 out of the 58 patients also agreed to participate in the CSF component. Included in the CSF study were 43 suicide attempters (15 men; mean age, 45 years; SD = 12.8; range, 22–69 and 28 women; mean age, 36 years; SD = 12.7; range 18–68). The same inclusion and exclusion criteria applied as described for Study I.

The patients included did not receive psychotropic drugs of the antidepressant or antipsychotic class during a washout period (mean, 21 days; SD = 13.6) after the suicide attempt during admission to the ward. A few patients in the CSF sample (n = 7, 16%) were treated prior to admission with antidepressant medication. The mean washout time for these patients was 34 days (SD = 14.1; range, 26–62 days).

A diagnostic assessment was conducted as described for Study I. For this sample, 95 % of the participants had at least one current axis I psychiatric diagnosis; 79 % of patients

satisfied the criteria for mood disorders (unipolar, major depressive disorder, single episode or recurrent, bipolar disorder, depressed or dysthymic disorder), 7 % for adjustment disorder and 5 % for anxiety disorders, one patient had a substance-use disorder and one an unspecified psychiatric disorder (not psychotic). Twenty percent of the patients had a comorbid substance-use disorder (mostly alcohol-use disorder). Among axis II diagnoses, 37 % of the patients satisfied the criteria for a personality disorder.

Somatic diagnoses in the sample: two patients had cardiovascular disease, three patients diabetes, one patient Crohn's disease, one patient celiac disease, and four patients suffered from chronic pain, NUD.

We used the Montgomery Åsberg Depression Rating Scale (MADRS) to assess the severity of depression (100).

We also included 20 healthy male volunteers (mean age, 29 years; SD = 5.0; range, 22–41). Healthy volunteers were all screened with an SCID interview conducted by a trained clinical psychiatrist to exclude any previous or current psychiatric disorders or any somatic conditions. All volunteers were screened for absence of psychiatric disorders in first-degree relatives. They were free from medication (total absence of psychotropic medication and no anti-inflammatory medication during the month previous to attendance), and they were also free from any substance-use disorder.

#### Study flowchart:

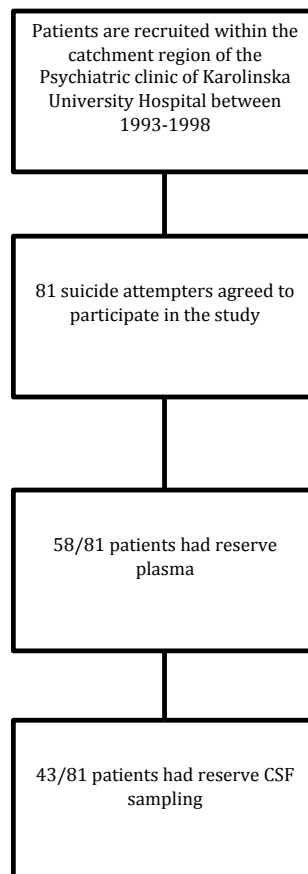


Table 3. Demographic and clinical data concerning the total study population.

<b>Variable</b>	<b>Plasma Studies I and II</b>	<b>CSF Study II</b>	<b>CSF Study III*</b>	<b>CSF healthy volunteers</b>
<b>Suicide attempters</b>	n = 58	n = 43	n = 39	n = 20
<b>Age (years) mean <math>\pm</math> SD, range</b>	37.2 $\pm$ 12.2, 18–69	39.0 $\pm$ 13.3, 18–69	38.6 $\pm$ 13.6, 18–69	28.9 $\pm$ 5.0, 22–41
<b>Age (years) mean <math>\pm</math> SD, range (male)</b>	39 $\pm$ 12.7, 20–69	45 $\pm$ 12.8, 22–69	45.6 $\pm$ 13.4, 22–69	28.9 $\pm$ 5.0, 22–41
<b>Age (years) mean <math>\pm</math> SD, range (female)</b>	36 $\pm$ 12, 18–68	36 $\pm$ 12.7, 18–68	35.5 $\pm$ 12.8, 18–68	–
<b>Gender (n=male)(%male)</b>	23, 40%	15, 35%	12, 31%	20, 100%
<b>BMI (kg/m<sup>2</sup>) mean, range</b>	24.6, 18.1–32.8	24.8, 18.2–32.8	24.7, 18.2–32.8	Not reported
<b>Depression as primary diagnosis</b>	44/58, 76%	33/43, 77%	30/39, 77%	-
<b>Personality disorder</b>	22/56**, 39 %	17/42**, 40 %	15/38**, 39 %	-
<b>Alcohol use disorder</b>	14/58, 24%	9/43, 21%	7/39, 18%	-
<b>Suicide method, violent (% yes)</b>	11/58, 19%	9/43, 21%	7/39, 18%	-

\* The duplicates of four patient samples regarding IL-6 showed high variability and were therefore excluded from the analysis (see also the biological analysis section – Study III.)

\*\*In the plasma cohort two recordings, and, in the CSF cohort, one recording of personality disorders were missing.

### 3.1.2 Subjects, Study IV

For this study we used a longitudinal design in which healthy participants were recruited for one of two physical exercise conditions, four days in a row of intensive exercise or moderate thrice weekly sessions during four weeks, based on an assessment of their habitual physical exercise level.

All participants were instructed to refrain from all physical exercise activity seven days before biological sampling was performed. Following the baseline sampling, participants were instructed to start their exercise according to a preformed standardized protocol. The post-exercise sampling was performed the day after the participants had finished the exercise program. We used paired sampling from blood and cerebrospinal fluid.

The study protocol was approved (Dnr 2014/1201-31/1) by the Regional Ethical Review Board in Stockholm, Sweden. All participants gave their written informed consent.

The recruitment was conducted by advertising on local student campuses and in research laboratories, and also on the internet at websites targeted at college students.

Inclusion criteria for the study were as follows:

- Men and women aged 18 years or older;
- Completely healthy with regard to both psychiatric and somatic disorders;
- A habitual exercise level adequate for either of the exercise protocols.

Exclusion criteria for the study were as follows:

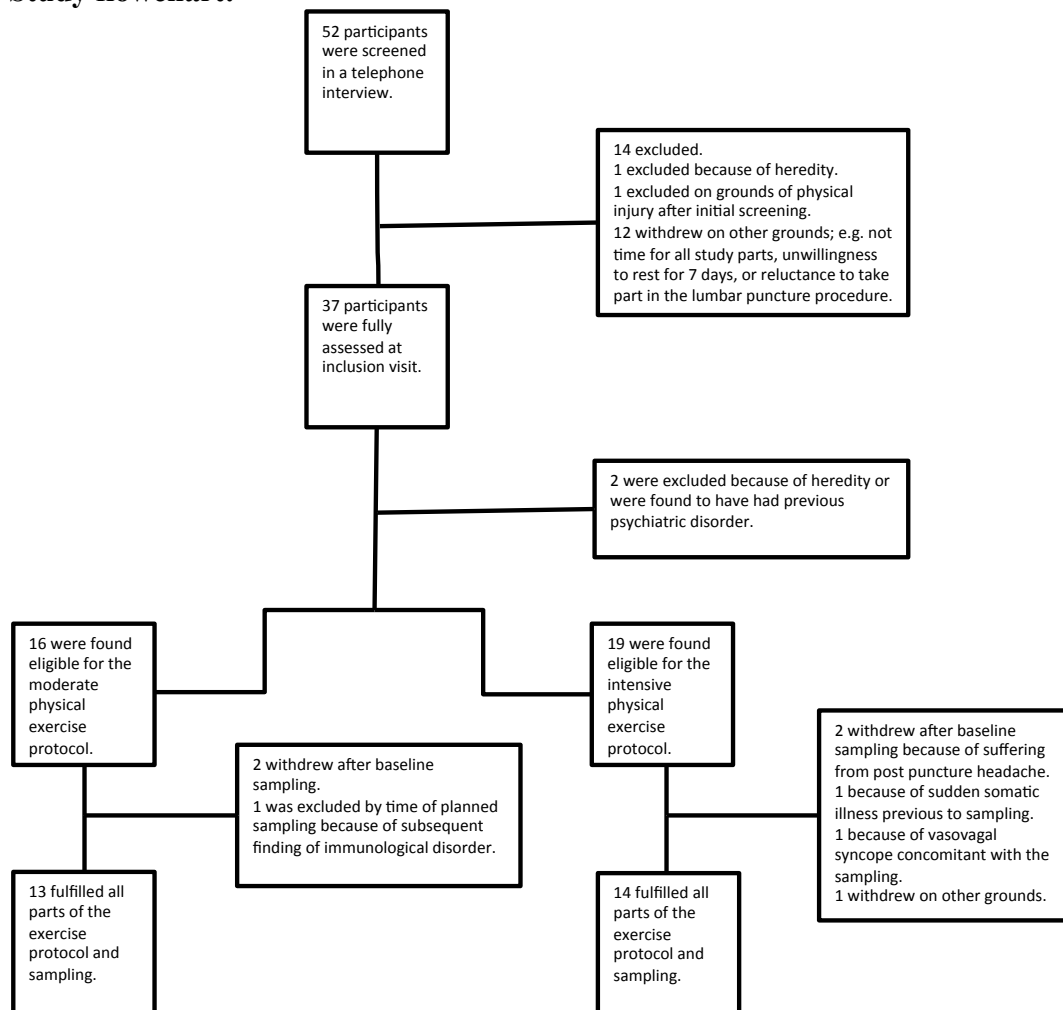
- An inadequate understanding of the study procedure and hence failure to provide informed consent;
- Past or current major psychiatric or physical disorder (with an emphasis on autoimmune or inflammatory disorders);
- Known heredity in first-degree relatives for severe psychiatric disorders, such as schizophrenia, bipolar disorder, major depression, and/or suicidal behavior;
- Any current physical hindrance that precludes partaking in the physical exercise protocol.

Participants contacted the researchers mainly through e-mail and were asked to provide a phone number. They were contacted initially for a telephone screening to determine their eligibility. Participants who were preliminarily determined to be eligible were booked for an inclusion visit with a trained clinical psychiatrist.

At the inclusion visit, the participants were asked to provide a full medical history. The diagnostic psychiatric MINI interview (130) was used to screen for common psychiatric disorders. The MINI interview screens for both current and past episodes of common psychiatric disorders. Participants were also screened and assessed for their current health status and a basic clinical examination was performed. The participants were also asked to fill out the Patient Health Questionnaire 9 (PHQ-9) (102) before and after exercise. The questionnaire was supposed to be filled out on the day of biological sampling. The PHQ-9 is a frequently used screening tool for depressive symptoms, and it is also a tool for following depressive symptoms over time.

Participants were deemed eligible for one of two physical exercise conditions.

### Study flowchart:



## 3.2 PSYCHOMETRIC INSTRUMENTS

### 3.2.1 SCID I and II

The Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (SCID) is a semi-structured interview (99). The interview is supposed to be done by a trained clinician with the purpose of determining the occurrence of diagnoses according to the Diagnostic and Statistic Manual of Mental Disorders (DSM). The SCID interview can assess diagnoses within the axis I disorders (SCID I), which are the main psychiatric syndromes, and diagnoses within the axis 2 disorders (SCID II), which are personality disorders. For our study, SCID interviews were conducted to assess diagnoses according to the, at the time, current version of DSM, the DSM III (99).

### 3.2.2 Montgomery-Åsberg Depression Rating Scale (MADRS)

The Montgomery-Åsberg Depression Rating Scale (MADRS) was originally designed to be a rating scale that could be used in clinical research, since the rating was supposed to be sensitive to change. The MADRS consists in a clinician-administered interview

where the clinician assesses depressed mood from ten separate items. The scale is widely used in scientific research and pharmaceutical studies (100).

### 3.2.3 Suicide Intent Scale (SIS)

Beck's Suicide Intent Scale (SIS) is an instrument consisting of 15 items. The different items are aimed at exploring the circumstances surrounding the suicide attempt. The items explore the patients' feelings and appreciation of the lethality of the suicide attempt (103). A two-factor model of the SIS was developed (factor 1: Lethal intent subscale; and factor 2: Planning subscale) and was used in this thesis (104).

### 3.2.4 Karolinska Scale of Personality

The Karolinska Scale of Personality (KSP) is constructed as a self-assessment questionnaire. The incentives behind the construction were the idea of measuring personality traits that are thought of as vulnerability factors in biological research and to gain a further understanding of underlying mechanisms in psychiatric disorders (105). Its construct was initially developed within psychopathy research, but it was subsequently tested in healthy individuals and was also used in schizophrenia and suicidal behavior research (105).

The original KSP inventory consists of 135 individual items that are grouped into 15 subscales. Each item is provided, as a statement that can be responded to using a four-point Likert-type scale ranging from "Does not apply at all" to "Applies completely". The psychometric properties of the KSP and its subscales, with regard to test-retest long-term stability, internal consistency and predictive ability, are generally reported as acceptable (106,107).

The KSP raw scores used in this thesis were transformed into T scores (population  $M = 50$ ,  $SD = 10$ ) based on an age- and gender-stratified Swedish normative sample (105). There is a strong intercorrelation between individual subscales and, to reduce multicollinearity, scales have been grouped into a four-factor structure proposed by Gustavsson and collaborators (106) which includes:

- 1) Neuroticism (including the subscales Socialization [negative loading], Somatic anxiety, Psychic anxiety, Muscular tension, Psychasthenia, Inhibition of aggression, Irritability, and Guilt);
- 2) Psychoticism (including the subscales Detachment and Suspicion)
- 3) Nonconformity (including the subscales Social desirability [negative], Indirect aggression, and Verbal aggression)
- 4) Extraversion (including the subscales Impulsiveness and Monotony avoidance).

This factor structure has been cross-validated by our research group, using a large cohort of suicide attempters, also including the patient sample used in Studies I–III (108) and is also used in this thesis (Study III). Our study focuses on the Extraversion factor, dominated by impulsiveness and sensation-seeking; see Table 4 for items included.

Table 4. Items from the KSP subscales, Impulsiveness and Monotony Avoidance. False (F) items indicate reverse scoring. Adapted from the Karolinska Scale of Personality (105).

Item row	KSP Impulsiveness items
(8)	I have a tendency to act on the spur of the moment without really thinking ahead.
(20)	When I have to make a decision, I "sleep on it" before I decide. (F)
(30)	I usually get so excited over new ideas and suggestions that I forget
(48)	I often throw myself too hastily into things.
(62)	I am a very particular person. (F)
(68)	I think it is quite right to describe me as a person who takes things as they come.
(81)	I usually "talk before I think".
(101)	When I'm about to make a decision, I usually make it quickly.
(113)	I take life easy.
(127)	I consider myself an impulsive person.
Item row	KSP Monotony Avoidance items
(2)	I am always keen on trying out things that are all new.
(22)	I like leading a quiet and organized life. (F)
(28)	I prefer people who come up with exciting and unexpected activities.
(44)	I have an unusually great need for change.
(54)	I try to get to places where things really happen.
(73)	I almost always have a desire for more action.
(84)	In a way I like to do routine jobs. (F)
(102)	I like doing things just for the thrill of it.
(109)	To be on the move, travelling, change and excitement - that's the kind of life I like.
(130)	When listening to the radio, I want it really loud, so that I can feel "turned on."

The KSP has more recently been revised and the updated construct is known as the Swedish Universities Scale of Personality (SSP). The purpose of the SSP was to improve psychometric properties item homogeneity and face validity as compared to the original KSP. The length of the scale as well as the same amount of questions for each subscale were considered to improve practicality (109). The SSP subscales were also designed as a supposedly pool of scales where single scales could be used for

research properties. The SSP contains 13 scales, and all subscales contain seven questions with a four-point response format ranging from “does not apply at all” to “applies completely.” Normative data were calculated from a random sample of 741 subjects living in Sweden. For Study IV, we included the SSP in the original inclusion procedure for future assessments on plausible correlations between personality traits and immune markers.

### 3.2.5 MINI interview

The MINI interview is a structured clinical interview developed to be used together with the clinician’s own assessment from the subject’s medical history. It is a short interview and has been validated with acceptable agreement toward a gold-standard SCID interview in assessing diagnoses according to the DSM or the International Classification of Diseases (ICD) (101). Because of its easiness of use and the short time for administration, it is widely used in clinical research. The interview determines the occurrence of affective disorders, anxiety disorders, substance use disorders, eating disorders or psychotic disturbances, previous or current.

### 3.2.6 PHQ-9

The Patient Health Questionnaire 9 (PHQ-9) is a tool intended to screen for depressive symptoms (102). It is also useful for following depressive symptoms over time. It measures nine symptom dimensions, based on current criteria for major depressive disorder. It also contains a tenth item that rates function. The score on each item ranges from 0 to 3, where 0 indicates no occurrence of a depressive symptom and 3 that the depressive symptom occurs most of the time, with only the nine symptom items being summed, with a possible total score ranging from 0 to 27 points.

### 3.2.7 Borg’s rating of perceived exertion (The Borg Scale)

The scale is commonly known as the Borg scale, named after the Swedish psychologist Gunnar Borg (110). Borg’s rating of perceived exertion (RPE scale) is an indicator of the accomplished level of peak exertion (110). The Borg scale has been validated and previous studies have reported very good levels of correlation with physiological variables of physical exertion (110); however, a recent meta-analysis concluded that the correlation levels might have been somewhat overrated previously and, in this report, the correlation is more moderate (0.62 for heart rate, 0.64 for %VO<sub>2</sub>max, and 0.72 for respiratory rate) (111). Recently, a study reported that there were no significant differences between physical exertion regulated by heart-rate monitoring or by using the RPE scale in a sample of healthy participants undertaking high-intensity interval training, suggesting that the RPE scale is a valid tool when prescribing exercise without clinical monitoring (112).



### **3.3 BLOOD SAMPLING PROCEDURE**

#### **3.3.1 Studies I–III**

Blood was sampled at the time of the suicide attempt during admission to the hospital. The sampling was performed after a night of fasting and bed rest. It was performed in the morning between 7:30 a.m. and 8:00 a.m..

Sampling was performed throughout the years 1993 to 1998, during which time the patients were recruited. It was done throughout every season. The study protocol assured that the same conditions were applied to all the samples. Blood was centrifuged within 5 min at room temperature ( $1000 \times g$  during 10 min). Plasma was collected and stored at  $-80^{\circ}\text{C}$  until measuring cytokine. The samples had never been thawed prior to the plasma immunoassay performed in 2010.

#### **3.3.2 Study IV**

Blood sampling was performed in a standardized manner between 7:30 a.m. and 9:00 a.m. All participants were instructed to refrain from physical exercise for seven days prior to baseline sampling. Participants had fasted since midnight and came to the clinic after a night of bed rest. Blood was drawn and collected in a BD vacutainer serum tube (9 ml) and allowed to clot for 30 minutes at room temperature before being centrifuged within an hour (2900 rpm/1692 rcf for 15 minutes). Due to an administrative error during sampling, three of the participants had no stored serum; therefore, the serum group consists of 24 participants, 12 in each exercise group. The same conditions were applied to all the samples. The aliquot samples had never been thawed prior to the analysis. Samples were collected between February and July, 2015.

### **3.4 CSF SAMPLING PROCEDURE**

#### **3.4.1 Studies II–III**

Lumbar punctures were performed at the end of the washout period in a standardized manner, after blood sampling, between 8:00 a.m. and 9:00 a.m.. Sampling was performed after a night of fasting and bed rest. CSF samples were collected from all seasons throughout the year between 1993 and 1998. A volume of 12 ml of CSF was drawn with the patient in the sitting position, the needle being inserted between lumbar vertebrae IV and V. The CSF was immediately centrifuged and stored at  $-80^{\circ}\text{C}$  until analyzed. The aliquot CSF samples had never been thawed prior to the immunoassay done in November, 2011.

#### **3.4.2 Study IV**

Lumbar punctures were performed in a standardized manner between 7:30 a.m. and 9:00 a.m.. All participants were instructed to refrain from physical exercise for seven days prior to baseline sampling. Participants had been fasting since midnight and came to the clinic after a night of bed rest. A volume of 25 ml of CSF was withdrawn with the patient in the sitting or lying position. An “atraumatic” Sprotte needle (22G, 0.70 mm, 70 mm or 90 mm) was used for sampling, the needle being inserted between

lumbar vertebrae III and IV or IV and V. The CSF was immediately centrifuged and the supernatant stored at -80 degrees C until analyzed. The aliquoted samples had never been thawed prior to the analysis. Samples were collected between February and July, 2015.

### **3.5 BIOLOGICAL ANALYSIS**

#### **3.5.1 Study I: Randox Biochip**

For the first study, we used a high-throughput automated biochip immunoassay system developed by Randox Laboratories Ltd (Crumlin, UK). The biochip has been extensively validated against gold-standard ELISA for several common cytokines (113).

Using a multiplex panel, we investigated the following cytokines and growth factors: IL-1- $\alpha$ , IL1- $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , Monocyte Chemotactic Protein-1 (MCP-1), Epidermal Growth Factor (EGF), and VEGF.

The Biochip Array technology is used to perform simultaneous quantitative detections of multiple analytes from a single sample, a so-called multiplex. The technology involves the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilized antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay is employed for the cytokine array. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and the concentration of analyte present in a patient sample or control was calculated from a calibration curve.

All the values obtained in this study were in the range of the standard/calibration curve. Since this was the high-sensitivity kit, each calibration begins at 0 pg/ml. The inter-, intra-assay variation is less than 10% according to the manufacturer. Quality control procedures were all implemented in the cytokine profiles that had passed predefined acceptance criteria to guarantee a high degree of precision.

#### **3.5.2 Study II: Mesoscale**

##### **3.5.2.1 VEGF**

Samples were run on two MSD Human VEGF 96-well plates (K151BMC-1; Gaithersburg, MD) and the results were pooled. The assay calibrator was diluted according to the manufacturer's recommendation, except for the standard curve starting at 1000 pg/ $\mu$ L.

Each well was blocked with 150  $\mu$ L Blocker C (provided) for 2h at room temperature (RT) on an orbital shaker (400 rpm). The plates were washed three times with 150  $\mu$ L/well of Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T). Diluent 7 (provided) was added at 25  $\mu$ L/well. Calibrator, samples, and controls were added at 25  $\mu$ L/well. The plate was sealed and incubated overnight at 4°C on an orbital shaker (400 rpm). At the end of the incubation, the wells were washed three times with PBS-T. After the last wash, detection antibody was added at 25  $\mu$ L/well. The plate was sealed

and incubated in the dark for 2 h at room temperature on an orbital shaker (400 rpm). At the end of the incubation, the plate was washed 3 times as before. 150 µl of the MSD 1X Read Buffer T was added to each well and the plate was measured immediately on the MSD Sector Imager 2400 plate reader.

#### 3.5.2.2 *IL-6 and IL-8*

Samples were run on two MSD Human Pro-inflammatory-4 II Ultra-Sensitive 96-well plates (K15025C-1). The samples were run according to the manufacturer's recommendation, with the following exceptions: standard points starting at 1000 pg/µL. Calibrator, samples and controls were incubated overnight at 4°C.

#### 3.5.3 Study III: Mesoscale

The samples were analyzed using two MSD Human Pro-inflammatory-4 II Ultra-Sensitive 96-well plates (K15025C-1). The samples were analyzed according to the manufacturer's recommendations, with the following exceptions: The standard points started at 1000 pg/µL; and the calibrator, samples and controls were incubated overnight at 4°C. The duplicates of four patient samples regarding IL-6 showed high variability and were therefore excluded from the analysis. A total of 39 patients are included in the CSF Results and Table sections. The inter-assay variation was 7.3%, and the intra-assay variations were 7.9% and 6.6%, respectively.

#### 3.5.4 Study IV: Cytometric bead-array (CBA)

Bead-based flow cytometric arrays have the capability to simultaneously and quantitatively measure multiple antigens or antibodies in small volumes of biological fluid. This technology is suitable when attempting to assess immune cell expression, such as cytokines and chemokines. The technology utilizes individual beads with an individual fluorescent characteristic for the detector to identify. The beads have their own individual set-up of analytes that can be assayed (114). There are two commercially available instruments that utilize this technology developed by Luminex and BD Biosciences. For this study, we used the instrument by BD Biosciences that also developed a flexible bead assay kit which lets the user customize the analytes that can be assessed in a multiplexed fashion (114).

##### 3.5.4.1 *CSF assay*

Expecting the levels of cytokines to be low in CSF, the CBA Enhanced Sensitivity Flex Set (BD, San Jose, CA, USA) was used for IL-2, IL-6, IL-8, IL-10, TNF- $\alpha$ , and IFN- $\gamma$ . VEGF was analyzed according to the protocol and kit described for serum, although without dilution using 50 µL CSF, as done with the other CSF samples. All samples (n = 54) were run in duplicates. Lyophilized standards for all analytes were pooled in a polypropylene tube and reconstituted with 4 mL of assay diluent for 15 minutes. The standard was serially diluted 1:3, 1:9 through 1:729 with an initial 40 µL reconstituted standard in 460 µL of assay diluent for the top standard and 400 µL of assay diluent in each tube, including a standard-free negative control. Capture beads were added to the sample and standard wells and incubated for 2 hours at RT. Detection reagents were pooled and diluted to 20 µL per test and incubated for 2 hours at RT. The plates were washed twice by adding 200 µL wash buffer and centrifuging at 500 rpm for 5 minutes

with unforced deceleration and discarding the supernatant. An Enhanced Sensitivity Detection Reagent (100 µL per test) was added and left to incubate for 1 hour at RT. Plates were centrifuged and the supernatant was discarded once more as described. Lastly, 150 µL of wash buffer was added and the plates were placed on a shaker to resuspend the beads before sample acquisition.

The samples were acquired using Beckman Coulter CyAn ADP (FACS; Beckman Coulter, CA, USA) at 488 and 635 nm and 300 events per bead and analyzed with the FCAP Array v3.0 software (Beckman Coulter, CA, USA).

#### 3.5.4.2 Serum assay

BD CBA Human Soluble Protein Flex Set (BD, San Jose, CA, USA) was used for quantifying serum levels of IL-2, IL-6, IL-8, IL-10, TNF- $\alpha$ , and IFN- $\gamma$ . The protocol was the same as for CSF, with the exception of a serum dilution of 1:4 for all samples.

VEGF was analyzed from serum and was thawed and diluted 1:5 with assay diluent from the BD CBA Human Soluble Protein Master Buffer Kit. All samples (n = 54) were run in duplicates. Lyophilized standards for all analytes were pooled in a polypropylene tube and reconstituted with 4 mL of assay diluent for 15 minutes. The standard was serially diluted 1:2, 1:4 through 1:256 with an initial 500 µL of top standard transferred and 500 µL of assay diluent in each tube including a standard-free negative control. Capture beads were pooled, washed with 0.5 mL of wash buffer and centrifuged at 200 g for 5 minutes before aspirating the supernatant, and diluted in Capture Bead Diluent for a final volume of 50 µL per test. Capture beads were added to sample and standard wells and incubated for 1 hour at RT. Detection reagents were also pooled, diluted with Detection Reagent Diluent for a volume of 50 µL per test and incubated for 2 hours at RT. After adding 150 µL of wash buffer and centrifuging at 500 rpm for 5 minutes (unforced deceleration), the supernatant was discarded and the beads were resuspended with 300 µL wash buffer.

### 3.6 STATISTICAL ANALYSIS

#### 3.6.1 Study I

The JMP 12 (SAS Institute, Inc., Cary, NC, USA) statistical package for Mac OS X was used for all statistical analyses. Figures presented were done with Stata/SE 12.1 for Mac.

We identified two male patients as multivariate outliers by using the Mahalanobis distance (115). The exclusion of these individuals did not affect the results, and they were included in all statistical analyses. Patient characteristics were described by using the mean, the median and the range for quantitative variables. The Shapiro–Wilk test was used to test data regarding normal distribution. Data for all cytokines displayed a skewness above 2. We used nonparametric statistics (Kruskal–Wallis test) in continuous variables for between-group comparisons; suicide victims vs. survivors.

Tests of nonparametric correlations were performed using Spearman's  $\rho$ . Regression analysis was performed to control for age, gender and body mass index (BMI).

Pearson's chi-square and Fisher's exact test were used for cross tabulations of categorical variables.

Alpha was set at 0.05.

### 3.6.2 Study II

The JMP 12 was used for all statistical analyses. Figures presented were done with Stata/SE 12.1.

We identified one patient as being both a univariate outlier (3.5 S.D. above the mean of the patients) and a multivariate outlier calculated using the Mahalanobis distance (115). This patient was excluded from all statistical analyses.

Both CSF VEGF and IL-8 levels were normally distributed, whereas CSF IL-6 was not normally distributed. The potential effect of the confounding factors was tested in linear regression models. The model consisted of age, gender, BMI, a comorbid diagnosis of personality disorder and alcohol-use disorder. Only age was a significant predictor of CSF VEGF levels in the model ( $t = 2.14$ ,  $p = 0.04$ ); other  $p$  values ranged between 0.25 and 0.82. The group comparisons were adjusted for age using linear regression.

With regard to plasma levels of VEGF and IL-8, one patient was identified as being both a univariate outlier (6 SD above the mean of the patients) and a multivariate outlier calculated using the Mahalanobis distance. This patient was excluded from the correlation analysis between CSF and plasma levels of VEGF and IL-8. Tests of parametric correlations were performed using Pearson's  $r$ . Tests of nonparametric correlations were performed using Spearman's  $\rho$ .

Alpha was set at 0.05.

### 3.6.3 Study III

The JMP 12 was used for all statistical analyses. Figures presented were done with Stata/SE 12.1.

The plasma and CSF IL-6 data were tested for normality using the Shapiro-Wilk test. To reduce skewness to the right, all of the data were logarithmically transformed for additional analysis. The quantitative population characteristics were described using means, standard errors, and quantiles.

The potential effect of confounds with regard to the log of plasma IL-6 was tested using a multivariate linear regression model. The models consisted of age, gender, BMI, depression severity (as measured by the MADRS), a comorbid diagnosis of personality disorder, and alcohol-use disorder. Only age significantly predicted plasma IL-6 in the model ( $t = 3.8$ ,  $p = 0.0004$ ); other  $p$  values ranged between 0.26 and 0.86. Furthermore, adding a diagnosis of somatic comorbidity to a separate model with age and

extraversion as predictors did not affect the significance of the model, and somatic comorbidity did not predict plasma IL-6 ( $p = 0.94$ ).

Similarly, the potential confound effect for the log of CSF IL-6 was tested using the same parameters; however, no significance was found for the tested confounds, and the  $p$  values ranged between 0.09 and 0.95. The sample storage time was not significantly correlated with IL-6 levels ( $p = 0.96$  for plasma,  $p = 0.43$  for CSF).

Tests of parametric correlations were performed using Pearson's  $r$ . Tests of non-parametric correlations were performed using Spearman's  $\rho$ .

A standard forced multiple regression analysis tested the association between plasma IL-6 levels and KSP personality factors, with the former as the dependent variable and the latter (neuroticism, psychoticism, extraversion, and nonconformity) and age as the independent variables. Group differences (violent vs. non-violent suicide attempts) were computed using one-way ANOVAs for the continuous variables.

Alpha was set at 0.05.

#### 3.6.4 Study IV

The JMP 12 statistical package for Mac OS X was used for all statistical analyses.

The assayed variables were heavily skewed and tested for normality using the Shapiro-Wilk test. Only post-CSF IL-8 was normally distributed ( $p = 0.44$ ). The quantitative sample characteristics were described using means, standard deviations, and range. The potential effect of confounds with regard to the assayed variables was tested using a multivariate regression model. The models consisted of age, gender, and BMI. No effect for confounds was determined to be significant.  $P$  values ranged between 0.21 and 0.92. Nonparametric statistics (Kruskal–Wallis test) were used when comparing continuous variables across groups pre-intervention (High vs. Low/Moderate habitual exercise levels). Since analytes were not normally distributed, tests of matched variables differences in means pre- and post-intervention in the total group and across groups (intensive or moderate exercise group) were performed using nonparametric statistics (Wilcoxon matched-pair signed-rank test). Tests of nonparametric correlations were performed using Spearman's  $\rho$ .

Alpha was set at 0.05.

### 3.7 ETHICAL APPROVAL

#### 3.7.1 Ethical considerations

Studies conducted on clinical populations require careful ethical considerations. For this clinical study, we recruited patients with a recent suicide attempt who were also taken to an inpatient service for further psychiatric treatment. The process of lumbar puncture is an invasive procedure with known side effects and, at the moment, is not done in routine psychiatric care. Furthermore, being part of this study meant that the

patients were also thoroughly assessed clinically using an extensive clinical assessment battery, a procedure potentially contributing to more distress.

We believe, however, that the value in the short run for the included patients consisted in the extensive, non-routinely conducted assessment with plausible benefits for the patients during follow-up care, as well as their having had an extensive medical assessment since routine variables were also assayed in the biological samples provided. We also believe that, in the long run, again from the patients' perspective, the value of investigating the intrathecal compartment in this context is of marked benefit since the CSF would arguably be a better fluid for investigating the milieu surrounding the brain. By doing this, the patients will hopefully contribute to our further understanding of the complex biological pathophysiology underlying these debilitating disorders.

### 3.7.2 Studies I-III

These studies were conducted in accordance with the Declaration of Helsinki. The study protocols were approved (Dnr 93-211) by the Regional Ethical Review Board in Stockholm, Sweden.

### 3.7.3 Study IV

The study was conducted in accordance with the Declaration of Helsinki. The study protocols were approved (Dnr 2014/1201-31/1) by the Regional Ethical Review Board in Stockholm, Sweden.

## 4 RESULTS AND DISCUSSION

### 4.1 STUDY I

#### 4.1.1 Cytokines and descriptive statistics

Patient age and BMI correlated positively with plasma IL-6 levels (Spearman's  $\rho = 0.44$  for age; Spearman's  $\rho = 0.32$  for BMI) and tended to correlate positively with VEGF levels (Spearman's  $\rho = 0.25$  for age; Spearman's  $\rho = 0.18$  for BMI). The correlation between storage time and VEGF levels was not significant (Spearman's  $\rho = -0.03$ ,  $p = 0.81$ ). There was no significant effect of the degree of depression (MADRS score), comorbid diagnosis of a personality disorder, or a substance-use disorder on the cytokine levels in the suicide attempters.

#### 4.1.2 Cytokine levels and suicide risk

Seven suicides (12 %) occurred during the follow-up time: three females (8.7 %) and four males (17 %). The major finding was that the mean VEGF distinguished between suicide completers (mean  $\pm$  SE) ( $14.6 \pm 7$  pg/mL) and survivors ( $24.9 \pm 2.6$  pg/mL) ( $n = 58$ ,  $p = 0.033$ , Kruskal–Wallis test) (Table 5).

Table 5. Nonparametric test for associations between inflammation markers in survivors vs. completers.

Cytokines mean $\pm$ SD pg/mL	Suicide attempters (n=51)	Suicide completers (n=7)	P value
IL 1- $\alpha$	$0.14 \pm 0.14$	$0.12 \pm 0.04$	0.52
IL1- $\beta$	$1.0 \pm 1.88$	$0.39 \pm 0.49$	0.33
IL-2	$1.16 \pm 1.33$	$0.44 \pm 0.84$	<b>0.037*</b>
IL-4	$1.61 \pm 1.22$	$1.28 \pm 0.60$	0.90
IL-6	$1.38 \pm 0.97$	$1.82 \pm 2.84$	0.30
IL-8	$2.20 \pm 1.78$	$1.80 \pm 1.23$	0.75
IL-10	$0.52 \pm 0.48$	$3.27 \pm 7.38$	0.18
VEGF	$24.9 \pm 19.5$	$14.58 \pm 6.03$	<b>0.033*</b>
IFN- $\gamma$	$1.04 \pm 0.93$	$1.69 \pm 1.51$	0.063
TNF- $\alpha$	$3.07 \pm 1.58$	$2.48 \pm 1.36$	0.11
MCP-1	$136.59 \pm 84.70$	$112.37 \pm 6.51$	0.53
EGF	$24.29 \pm 17.97$	$24.97 \pm 21.55$	0.88



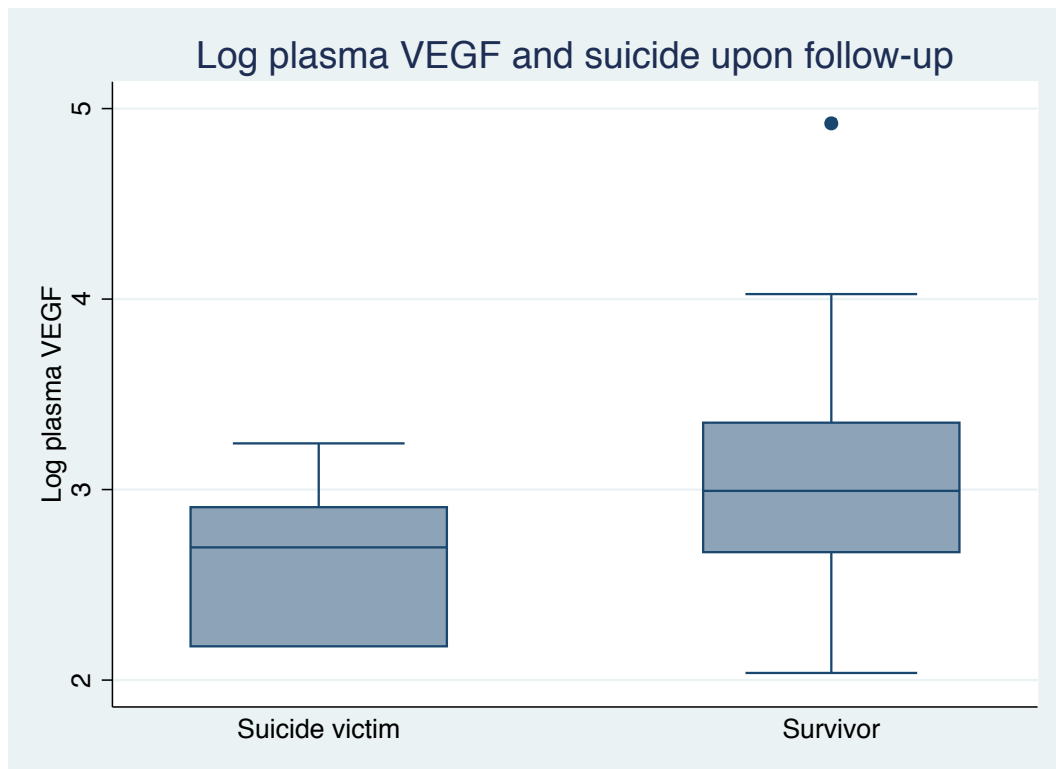


Figure 2. Box plot on log plasma VEGF at baseline across suicide completers vs. survivors upon follow-up.

When age, gender, and BMI were used as covariates in a regression analysis, the regression model with VEGF was significant ( $n = 58$ ,  $\chi^2 = 10$ ,  $df = 4$ ,  $p = 0.040$ ). VEGF was a significant predictor of suicide ( $p = 0.045$ ). Suicide victims had lower levels of IL-2 (Mean + SE) ( $0.4 \pm 0.49$  pg/mL) compared to survivors ( $1.2 \pm 0.18$  pg/mL) ( $n = 58$ ,  $p = 0.037$ , Kruskal-Wallis test). When age, gender, and BMI were used as covariates in a regression analysis, the regression model with IL-2 did not remain significant ( $n = 58$ ,  $\chi^2 = 6.8$ ,  $df = 4$ ,  $p = 0.14$ ). IL-2 showed a trend to being a predictor of suicide ( $p = 0.099$ ). There was a trend toward higher IFN- $\gamma$  levels in suicide victims compared to survivors ( $n = 58$ ,  $p = 0.063$ , Kruskal-Wallis test).

#### 4.1.3 Cytokines and suicide intent

Plasma VEGF tended to be negatively correlated with the Planning subscale of SIS (Spearman's  $\rho = -0.24$ ,  $p = 0.076$ ). Plasma IFN- $\gamma$  showed a significant positive correlation with SIS (Spearman's  $\rho = 0.26$ ,  $p = 0.048$ ) and with the Planning subscale (Spearman's  $\rho = 0.28$ ,  $p = 0.038$ ). IL-2 did not correlate with suicide intent.

#### 4.1.4 Discussion

The rationale for the first study was to determine how proinflammatory cytokines could be predictive and how they varied between suicide victims vs. survivors in a cohort of suicide attempters. We approached this question by using a longitudinal design where we conducted a follow-up regarding cause of death with our outcome measure being completed suicide.

The main finding in this study was that patients with a recent suicide attempt who, at follow-up had committed suicide, had significantly lower plasma levels of VEGF at the time of inclusion. We could also report that suicide victims had lower levels of IL-2, a finding that however did not reach significance in our regression model. Furthermore IFN- $\gamma$  levels tended to be higher in suicide victims. VEGF also tended to be negatively correlated with the planning subscale of the SIS, i.e., the more planned the suicide attempt, the lower the levels of VEGF.

The main finding regarding low plasma VEGF levels predicting complete suicide is interesting in the context of the neurotrophic hypothesis of depression and the theoretical discussion regarding VEGF as a possible trait marker for treatment resistance as suggested by, for example, Halaris and collaborators. Namely, while low VEGF levels at baseline in this sample, might constitute a more severe subgroup even within this high-risk cohort of suicide attempters, they might explain why these subjects had a less fortunate outcome.

Furthermore, our other findings regarding IL-2 and IFN- $\gamma$  are in line with other research in this field (87–89). Low peripheral levels of IL-2 have been reported previously, and so have increased peripheral levels of soluble IL-2 receptor (sIL-2R) in suicide attempters (116). Since IL-2 and sIL-2R binds into a complex that is internalized with lysosome degradation, elevated sIL-2R may explain the low levels seen in suicide attempters compared to controls in some studies (117,118).

Based on the above, we could propose a role for plasma VEGF as a plausibly relevant new biomarker for both prediction and as a trait marker. However, this obviously needs to be replicated in future studies.

## **4.2 STUDY II**

### **4.2.1 CSF immune markers and suicide attempters**

CSF VEGF and IL-8 levels were significantly lower in suicide attempters (VEGF mean, 3.7; pg/mL, IL-8 mean, 17.1 pg/mL) compared to controls (VEGF mean, 5.0 pg/mL; IL-8 mean, 25.6 pg/mL) (t-ratio = -3.76;  $p = 0.0004$ ; t-ratio = -5.82;  $p < 0.0001$ , both adjusted for age) (Fig. 3a and 3b).

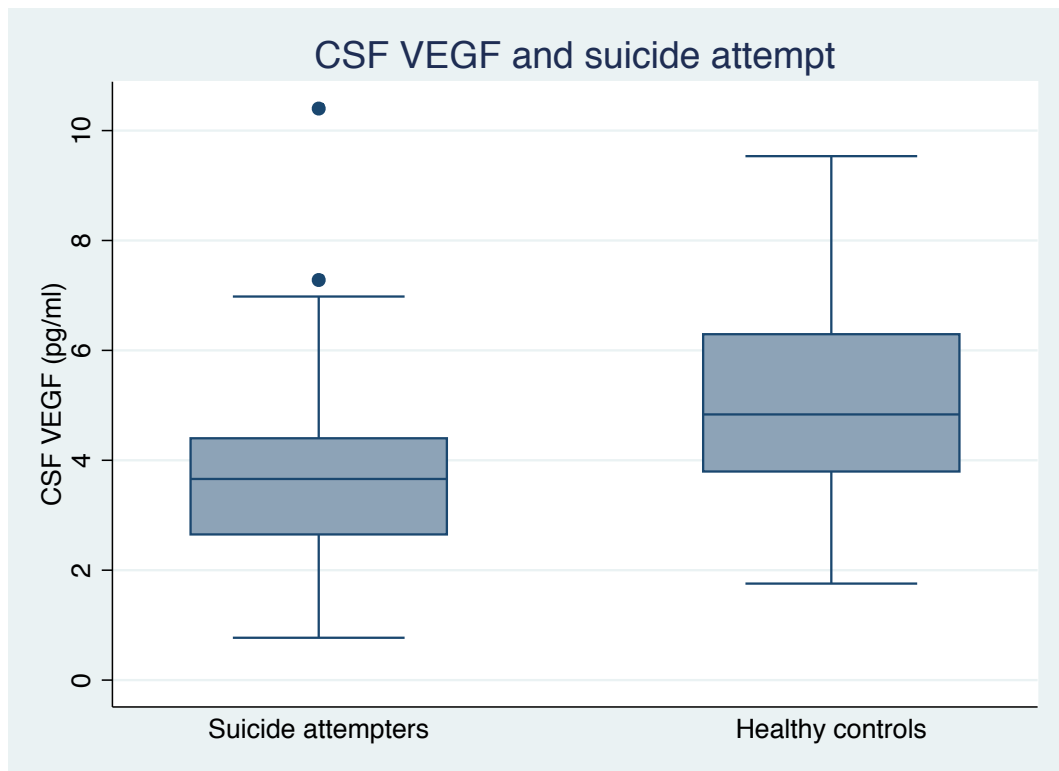


Figure 3a. Box plot on CSF VEGF in suicide attempters and healthy controls.

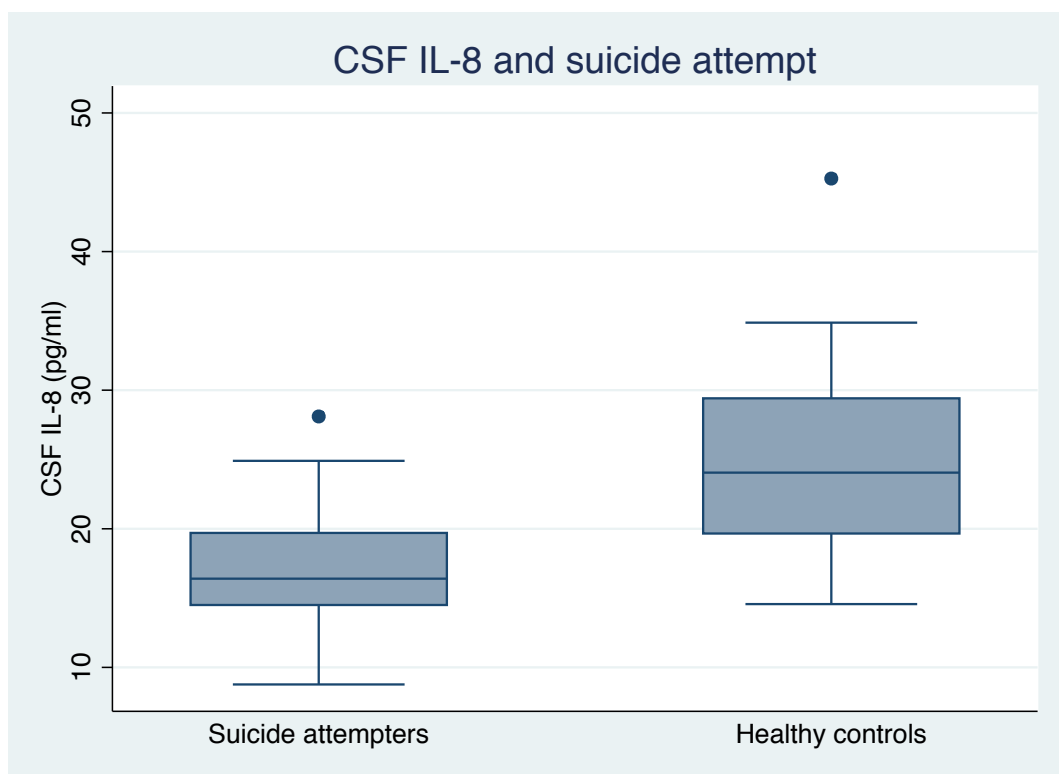


Figure 3b. Box plot on CSF IL-8 in suicide attempters and healthy controls

#### 4.2.2 CSF VEGF and depression severity

CSF VEGF levels showed a significant negative correlation with depression severity as measured with the MADRS ( $r = -0.31$ ;  $p = 0.049$ ) (Fig. 4). A correlation analysis of CSF IL-8 levels and MADRS was not significant ( $r = -0.13$ ,  $p = 0.40$ ).

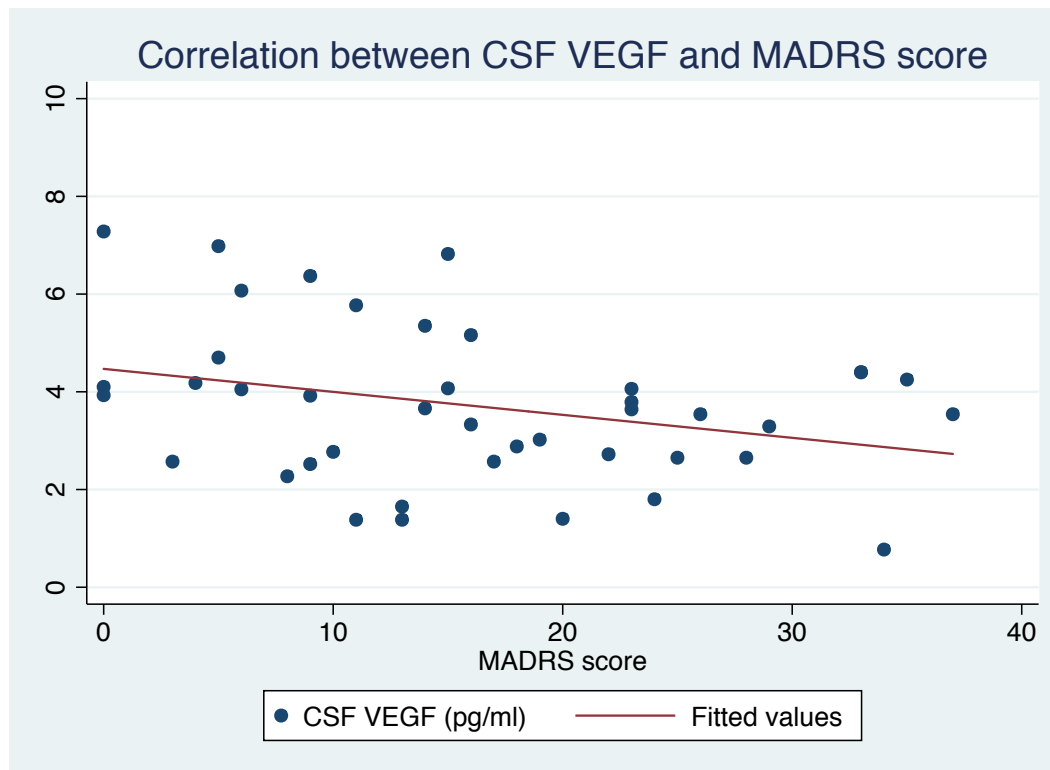


Figure 4. Correlation between CSF VEGF and MADRS.

#### 4.2.3 Correlation between immune markers

In suicide attempters, VEGF tended to be positively correlated with IL-8 levels ( $r = 0.29$ ,  $p = 0.066$ ). Interestingly, the levels of CSF VEGF and IL-8 did not correlate with plasma levels of VEGF and IL-8 in suicide attempters (Spearman's  $\rho = 0.04$ ,  $p = 0.80$ ; Spearman's  $\rho = 0.06$ ,  $p = 0.70$ ). CSF IL-6 levels did not differ in suicide attempters (mean 1.1 pg/mL) compared to controls (0.9 pg/mL) ( $t$ -ratio = 0.91,  $p = 0.37$ , adjusted for age).

#### 4.2.4 Discussion

The rationale for the second study was to determine cytokines and VEGF in the CSF in suicide attempters compared to healthy controls, in order both to assess whether we in our sample could replicate results from previous studies, and also whether potentially new findings in our sample could generate new information regarding biomarkers in suicide research.

The main finding was that suicide attempters had lower levels of VEGF and IL-8 in the CSF as compared to healthy controls. VEGF and IL-8 levels in the CSF did not correlate with peripheral levels. In our study, CSF IL-6 was not higher in suicide attempters compared to controls, inconsistent with the findings by Lindqvist and collaborators (63).

Our results indicate that low CSF VEGF and IL-8, might in this context be biomarkers regarding risk for suicide attempt. This finding is not readily interpretable, but speculatively, the low CSF VEGF levels could indicate a lack of neurotrophic support.

The lack of correlation between compartments suggests that this plausible down-regulation is segregated from the peripheral source. However, we did not include other neurotrophins or markers regarding the HPA axis in our assay, which excludes the possibility to determine whether this is selective for VEGF. We can neither rule out the possibility that our finding represents an epiphenomenon, which also could be the case regarding the finding relating to IL-8.

Chemokines are immune-competent proteins that signal to the immune system to recruit immune cells to the site of action during an immune challenge. IL-8 is one of the best known chemokines (about 50 human chemokines are described) that is important in recruiting neutrophils to target sites. IL-8 also seems to be of importance as a vascular signal together with VEGF and also when it comes to inflammation resolution, depending on the circulating levels of IL-8. High levels are reported to be considered anti-inflammatory, inhibiting further adhesion of neutrophils and repeated activation of IL-8 seems to attenuate the inflammation response through receptor down-regulation (119). IL-8 is also a pleiotropic molecule and has been reported to be proangiogenic, whereby an association has been previously reported between IL-8 and an increase in VEGF and its receptor VEGFR-2 via a Nuclear Factor kappa B (NFkB)-mediated pathway (120).

In psychiatric research, the reports concerning IL-8 vary between studies. Low peripheral IL-8 levels were previously associated with depression in a population-based sample (121). IL-8 in the CSF was previously described as being positively correlated with alexithymia and anxiety in patients with a non-inflammatory neurological disorder (122). A majority of the patients included in that study had a diagnosis of neurological pain, which could explain partly, the reported association with elevated chemokine levels. After our report, IL-8 has also been reported to be negatively correlated to anxiety in a cohort of suicide attempters (123). This study raises further questions about the role of IL-8 as a marker for a phenotypic subgroup with comorbid anxiety, i.e. symptoms that increase the clinical severity and constitute a statistical risk for recurrence of suicidal behavior (124).

Based on this study, a role for both CSF VEGF and IL-8 as relevant biomarkers for suicidal behavior could be suggested. Whether this is a state or trait marker, or a marker that may distinguish suicide attempters from other psychiatrically relevant comparison groups can not be ruled out and needs further clarification in future studies. These markers are part of innate immunity, suggesting that mechanisms within innate immunity should be more thoroughly elucidated.

### **4.3 STUDY III**

#### **4.3.1 Plasma and CSF IL-6 levels**

The plasma and CSF IL-6 results from the cytokine analyses are presented in Table 6. The correlation between the untransformed data of these levels in the patient sample was not significant ( $r = 0.15$ ,  $p = 0.39$ ).

Table 6. IL-6 levels in plasma and CSF.

Variable	Plasma IL-6	CSF IL-6
<b>Suicide attempters</b>	n = 58	n = 39
<b>Levels (pg/mL), mean <math>\pm</math> SEM</b>	1.43 $\pm$ 0.17	1.0 $\pm$ 0.15
<b>Range</b>	0.23-8.15	0.17-3.7
<b>Median, Quantile (0.1, 0.9)</b>	1.12, 0.30, 3.0	0.67, 0.33, 2.27

#### 4.3.2 Plasma and CSF IL-6 levels and KSP personality factors

The plasma IL-6 levels were significantly and positively correlated with the KSP personality factor extraversion ( $r = 0.48$ ,  $p < 0.0001$ ) (Fig. 5). In contrast, CSF IL-6 levels were not significantly correlated with any personality factor (Table 7).

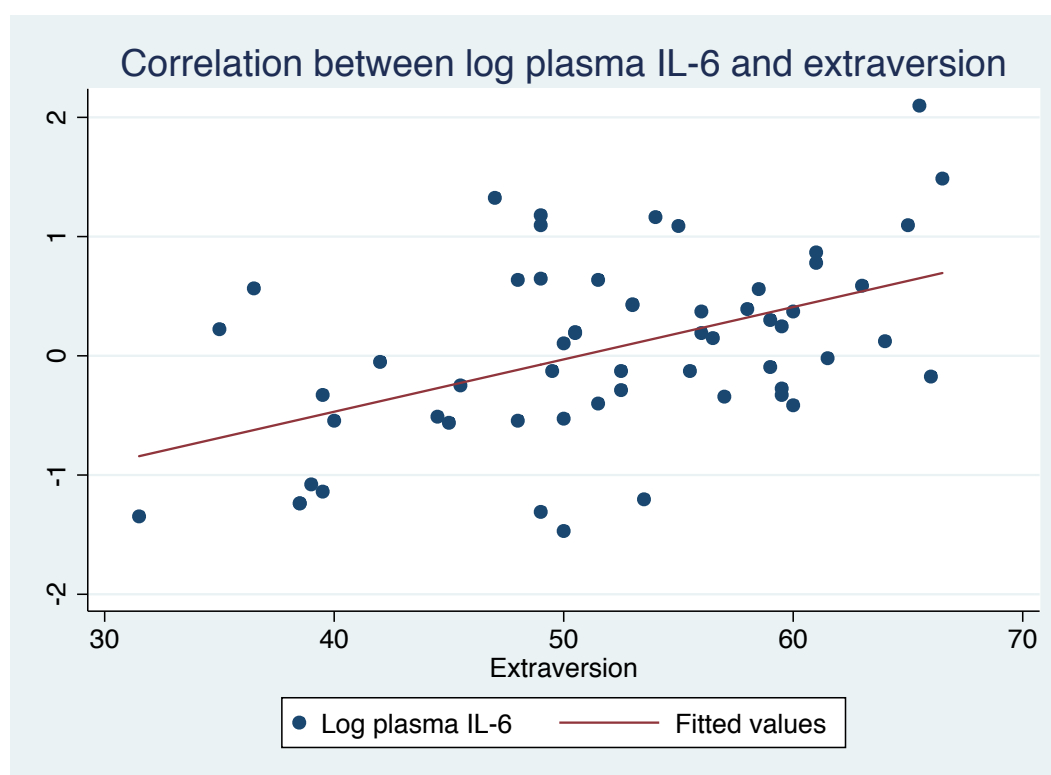


Figure 5. Correlation between log plasma IL-6 and extraversion

Table 7. Pearson's  $r$  correlation between personality factors and plasma and CSF IL-6 levels (log) \*\* $p < 0.0001$ .

Variable	Plasma IL-6	CSF IL-6	Neuroticism	Psychoticism	Extraversion	Nonconformity
<b>Neuroticism</b>	-0.20	-0.09	1	0.45**	0.01	0.08
<b>Psychoticism</b>	-0.05	-0.04	0.45**	1	0.03	-0.11
<b>Extraversion</b>	0.48**	0.25	0.01	0.03	1	0.18
<b>Nonconformity</b>	0.14	-0.10	0.08	-0.11	0.18	1

A standard linear regression model was used with the four KSP personality factors and age to predict plasma IL-6 levels. The regression model was significant: adjusted R-square = 0.41,  $F = 8.8$ ,  $DF = 5$ ,  $p < 0.0001$ . Extraversion and age were independent and significant predictors of plasma IL-6 levels (Table 8).

Table 8. Personality traits as predictors of plasma IL-6 in suicide attempters. R square = 0.46 ( $F$  ratio = 8.8,  $DF = 5$ ,  $p < 0.0001$ ).

Variable	t ratio	p value
<b>Extraversion</b>	4.38	0.0001
<b>Psychoticism</b>	0.23	0.82
<b>Neuroticism</b>	-0.49	0.63
<b>Nonconformity</b>	0.73	0.47
<b>Age</b>	4.22	0.0001

#### 4.3.3 Plasma and CSF IL-6 levels and KSP extraversion subscales

In the next step, a correlation analysis between the KSP subscales for extraversion (impulsiveness and monotony avoidance) and the plasma and CSF IL-6 levels was performed. Plasma IL-6 was significantly correlated with impulsiveness ( $r = 0.39$ ,  $p < 0.01$ ; Fig. 6a) and monotony avoidance ( $r = 0.36$ ,  $p < 0.01$ ; Fig. 6b), whereas CSF IL-6 was correlated with monotony avoidance ( $r = 0.35$ ,  $p < 0.05$ ; Fig. 6c). The correlation between CSF IL-6 and impulsiveness was not significant.

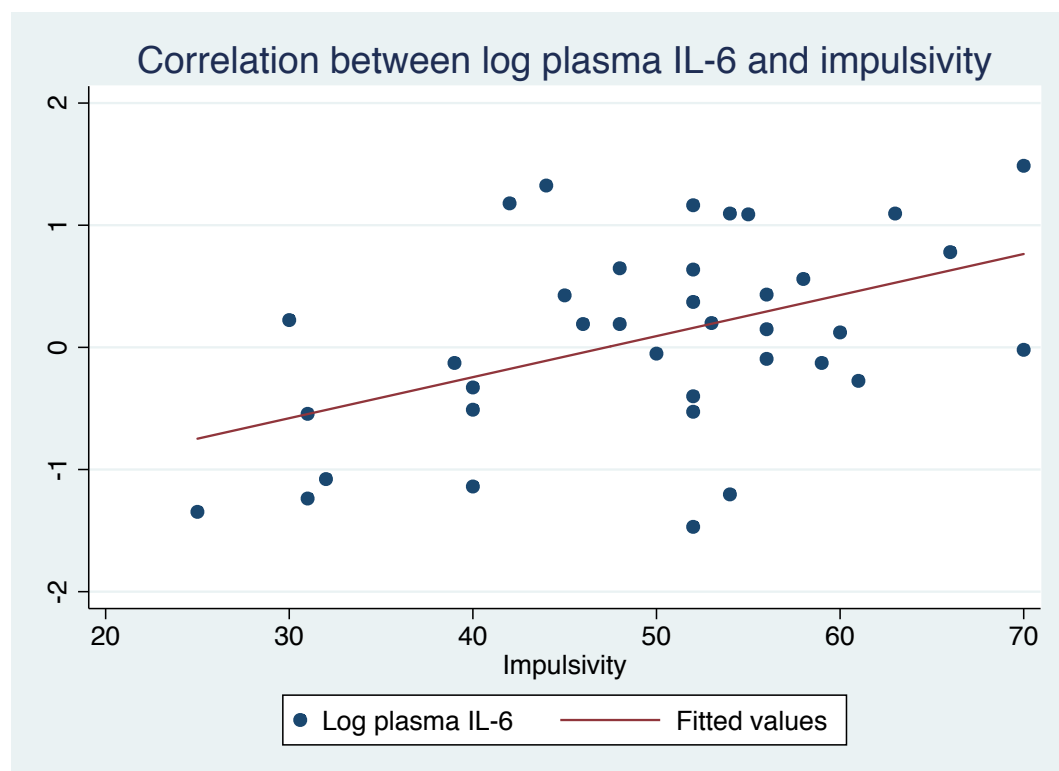


Figure 6a. Correlation between log plasma IL-6 and impulsiveness.

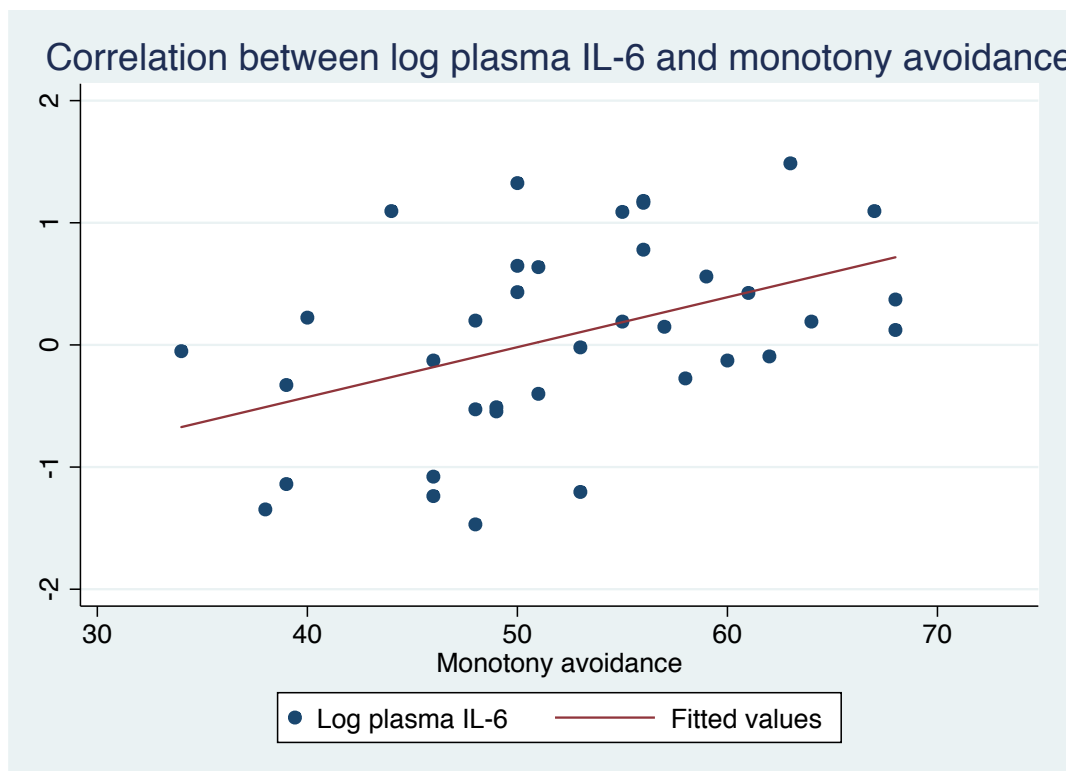


Figure 6b. Correlation between log plasma IL-6 and monotony avoidance.

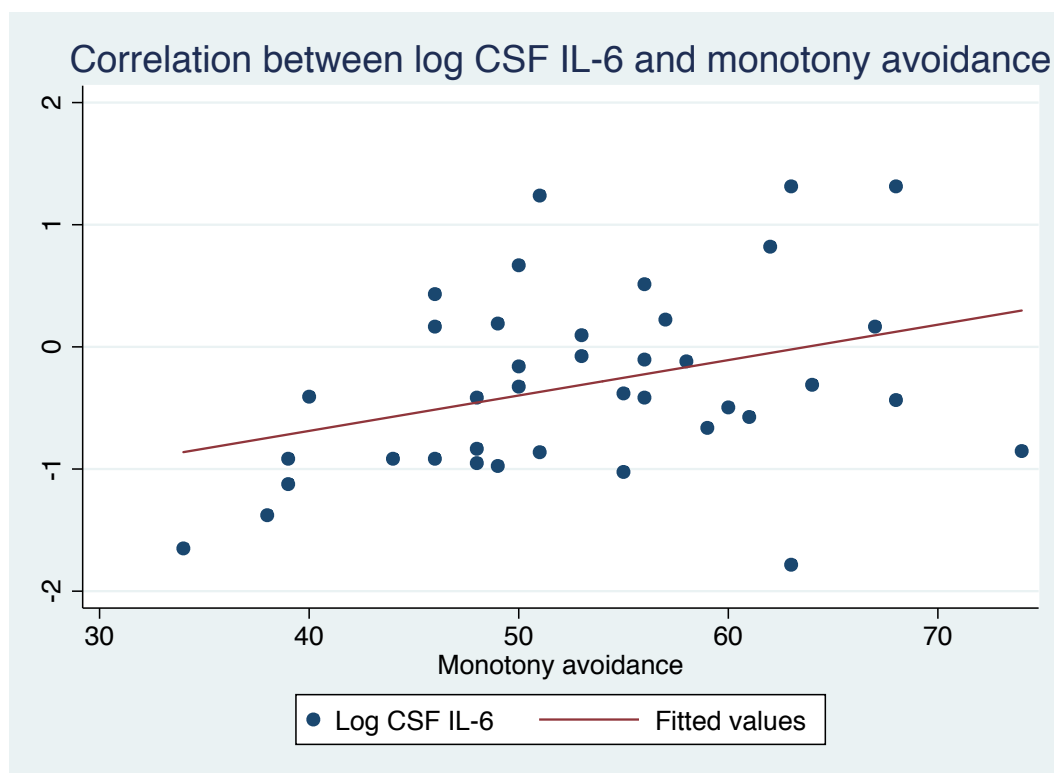


Figure 6c. Correlation between log CSF IL-6 and monotony avoidance.

#### 4.3.4 Plasma, CSF IL-6 and personality traits with regard to suicide attempt method

Plasma and CSF IL-6 levels tended to be higher among suicide attempters who had used a violent method than among the nonviolent attempters ( $F = 3.51$ ,  $p = 0.07$ ;  $F =$



2.4,  $p = 0.13$ , respectively). After adjusting for age and gender, the choice of a violent suicide attempt tended to be associated with higher plasma IL-6 levels ( $p = 0.051$ ).

Significant differences were not observed with regard to the extraversion scores for violent and nonviolent suicide attempters ( $p = 0.55$ ).

The regression model with plasma IL-6 as the dependent variable and age, choice of suicide attempt method, extraversion and the interaction between choice of method and extraversion did not reveal a significant interaction term ( $p = 0.13$ ). When the non-significant interaction term was removed from the model, it was significant (adjusted R-square = 0.45,  $F = 16.9$ ,  $DF = 3$ , and  $p < 0.0001$ ). Extraversion and age significantly predicted plasma IL-6 levels, whereas the choice of method tended to be significant ( $p = 0.07$ ).

#### 4.3.5 Discussion

The third study that is part of this thesis was designed to determine the potential effects of plausible endophenotypes determinable within our sample in relation to IL-6. The rationale for this was the finding that our results differed from those in previous studies with regards to IL-6 in relation to suicidal behavior, and also from findings in previous reports that IL-6 was also associated with depression in patients. Thus we could report that IL-6, in both plasma and the CSF, correlated with personality traits dominated by impulsivity or sensation-seeking in suicide attempters. We also saw that IL-6 tended to be higher, in both CSF and plasma, in suicide attempters who used a violent method, a finding consistent with a previous study conducted by Lindqvist and collaborators (63).

We think our results are theoretically interesting, since they combine both earlier research on signs of aberrant inflammation and personality traits and research regarding features that may predispose to suicide risk, where the same traits from both research paths report on impulsivity as an important measure. So, adding to the complexity, we used endophenotypes determinable from the initial assessments and studied how these may influence IL-6. Based on this study, we could suggest the need to place an emphasis on personality measures, perhaps best done by using neurocognitive test batteries when undertaking biomarker studies.

### 4.4 STUDY IV

#### 4.4.1 Sample characteristics

The final sample consisted of 27 participants (12 men, mean age = 30.2, s.d. = 9.1, range = 22–53; 15 women, mean age = 27.5, s.d. = 6.7, range = 21–45). The characteristics of the study participants in the both exercise groups are presented in Table 9. There were no statistically significant differences between the exercise groups concerning gender distribution, age, or BMI ( $p$  values ranged between 0.45 and 0.86).

All participants were screened negative for both a history of psychiatric disorder and any current psychiatric disorder. They were also screened negative for somatic morbidity, and routine clinical variables were all within normal reference values.

Participants were all nonsmokers, no self-reported history of substance-use disorder, and none were on any regular medication. Although not collected in all individuals, PHQ-9 was available for 15 participants at baseline and 17 participants post exercise (Table 9).

Table 9. Descriptive statistics concerning the study population.

<b>Variable</b>	<b><i>Total study group, n = 27</i></b>	<b><i>Intensive exercise group, n = 14</i></b>	<b><i>Moderate exercise group, n = 13</i></b>
<b>Gender (n = male/female)</b>	12/15	6/8	6/7
<b>Age (years) mean <math>\pm</math> SD, range</b>	28.7 $\pm$ 7.9, 21–53	28.1 $\pm$ 8.9, 22–53	29.2 $\pm$ 6.8, 21–45
<b>Age (years) mean <math>\pm</math> SD, range (male)</b>	30.2 $\pm$ 9.1, 22–53	33.0 $\pm$ 12.3, 23–53	27.3 $\pm$ 3.7, 22–33
<b>Age (years) mean <math>\pm</math> SD, range (female)</b>	27.5 $\pm$ 6.7, 21–45	24.5 $\pm$ 2.1, 22–28	30.9 $\pm$ 8.7, 21–45
<b>BMI (kg/m<sup>2</sup>) mean, range</b>	22.6 $\pm$ 2.6, 18.9–29.6	22.9 $\pm$ 2.7, 19.6–29.6	22.2 $\pm$ 2.5, 18.9–28.0
<b>PHQ-9 at baseline, Mean <math>\pm</math> SD, range</b>	1.9 $\pm$ 3.2, 0–11 (n = 15)	0.7 $\pm$ 1.2, 0–3 (n = 6)	0.4 $\pm$ 0.7, 0–2 (n = 8)
<b>PHQ-9 post exercise, Mean <math>\pm</math> SD, range</b>	1.2 $\pm$ 2.0, 0–7 (n=17)	2.8 $\pm$ 3.8, 0–11 (n=9)	2 $\pm$ 2.5, 0–7 (n=9)

#### 4.4.2 Exercise habits and completion

All participants included rated their habitual level of exercise based on the number of weekly sessions and the intensity of exercise, limited to the previous six months. In the intensive group, all participants were determined to have a high level of habitual exercise and, in the moderate group, six participants were determined to be sedentary, while the rest had a current moderate level of exercise. The habitual exercise habits and the number of completed exercise sessions are described in Table 10.

Table 10. Habitual exercise level at baseline and exercise sessions completed.

<b>Variable</b>	<b><i>Intensive exercise group, n = 14</i></b>	<b><i>Moderate exercise group, n = 13</i></b>
<b>Habitual exercise level at baseline (Low-Moderate-High)</b>	High level, n = 14	Low level, n = 6 Moderate level, n=7
<b>Exercises completed (Intensive group, 4 sessions intended, moderate group, 12 sessions intended)</b>	4 sessions completed, n = 14	12 sessions completed, n = 9 13 sessions completed, n = 3 14 sessions completed, n = 1

#### 4.4.3 CSF immune markers and habitual exercise level

The CSF levels of the assayed analytes that were above the detection limit are presented in Table 11.

Baseline CSF IL-8 levels, pre-intervention, tended to be higher among the participants with a high habitual exercise level ( $p = 0.06$ , Kruskal-Wallis test).

Baseline CSF IL-6 levels, pre-intervention, were not significantly different in the groups with different habitual exercise levels ( $p = 0.7$ , Kruskal-Wallis test).

Table 11. IL-6 and IL-8 levels in CSF in total and across groups.

<b>Variable</b>	<b><i>Total (n = 27)</i></b>	<b><i>Intensive (n = 14)</i></b>	<b><i>Moderate (n = 13)</i></b>
<b>Pre CSF IL-6 (pg/mL), mean <math>\pm</math> SD</b>	1.99 $\pm$ 2.62	1.55 $\pm$ 0.95	2.46 $\pm$ 3.66
<b>Post CSF IL-6 (pg/mL), mean <math>\pm</math> SD</b>	2.46 $\pm$ 2.86	2.34 $\pm$ 1.87	2.59 $\pm$ 3.74
<b>Pre CSF IL-8 (pg/mL), mean <math>\pm</math> SD</b>	35.9 $\pm$ 12.7	40.2 $\pm$ 14.6	31 $\pm$ 8.74
<b>Post CSF IL-8 (pg/mL), mean <math>\pm</math> SD</b>	43.2 $\pm$ 13.6	46.7 $\pm$ 16.0	39.4 $\pm$ 9.7

#### 4.4.4 CSF immune marker variability after exercise

The pre- and post-variability for CSF IL-8 was significant for the group total ( $p = 0.0002$ , Wilcoxon matched-pair signed-rank test); and for the testing across groups, intensive or moderate ( $p = 0.05$ ,  $p = 0.005$ , Wilcoxon matched-pair signed-rank test) (Fig. 7).

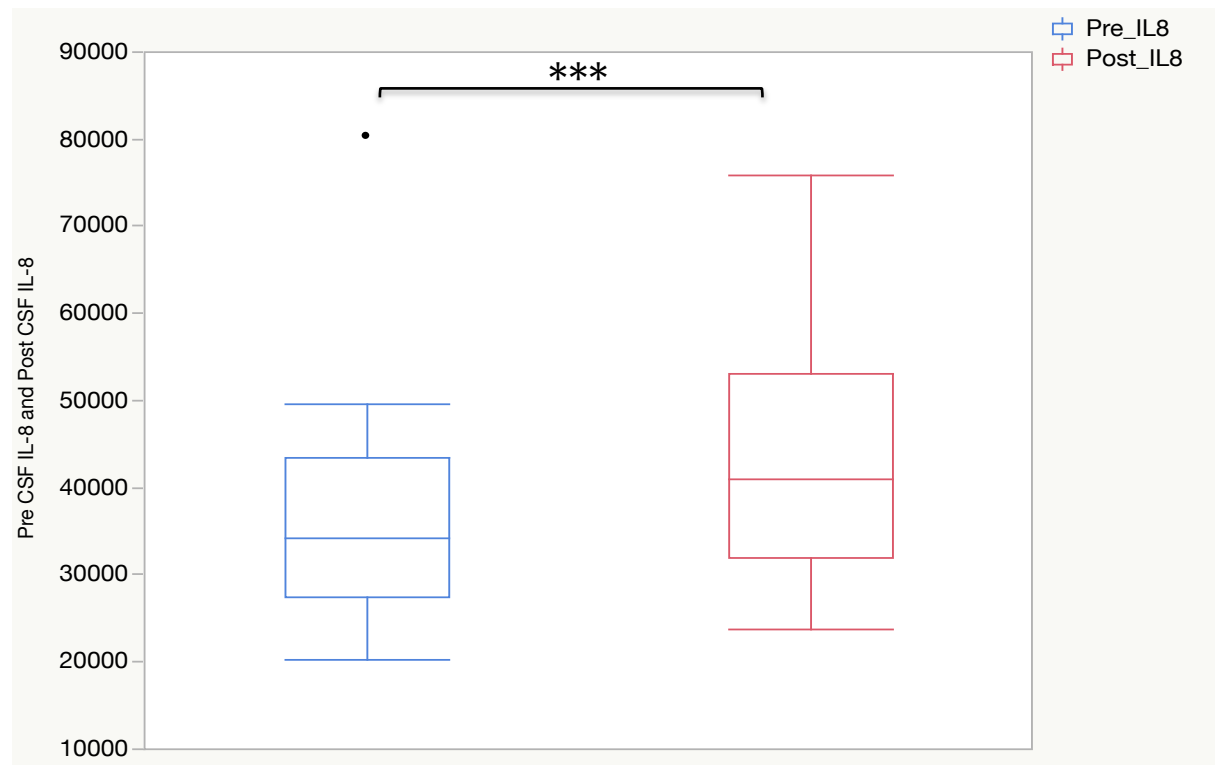


Figure 7. Box plot over CSF IL-8 (fg/mL) pre- and post-exercise. (\*\*  $p < 0.0005$ ).

The pre- and post-variability for CSF IL-6 was not significant for the group total ( $p = 0.13$ , Wilcoxon matched-pair signed-rank test); or for the testing across groups, intensive or moderate ( $p = 0.24$ ,  $p = 0.24$ , Wilcoxon matched-pair signed-rank test).

#### 4.4.5 Serum immune markers and habitual exercise level

The serum levels of the assayed analytes that were above the detection limit are presented in Table 12.

Baseline serum IL-6, IL-8, and VEGF levels, pre-intervention were not significantly different according to the habitual exercise level ( $p$  values ranged between 0.44 and 0.62, Kruskal-Wallis test).

Table 12. IL-6, IL-8 and VEGF levels in serum by total and across groups.

Variable	Total (n = 24)	Intensive (n = 12)	Moderate (n = 12)
<b>Pre IL-6 (pg/mL), mean <math>\pm</math> SD</b>	0.21 $\pm$ 0.33	0.12 $\pm$ 0.16	0.30 $\pm$ 0.43
<b>Post IL-6 (pg/mL), mean <math>\pm</math> SD</b>	0.42 $\pm$ 0.53	0.30 $\pm$ 0.31	0.55 $\pm$ 0.67
<b>Pre IL-8 (pg/mL), mean <math>\pm</math> SD</b>	4.18 $\pm$ 3.12	4.02 $\pm$ 2.07	4.35 $\pm$ 4.00
<b>Post IL-8 (pg/mL), mean <math>\pm</math> SD</b>	5.23 $\pm$ 3.13	4.42 $\pm$ 2.59	6.04 $\pm$ 3.51
<b>Pre VEGF (pg/mL), mean <math>\pm</math> SD</b>	10.7 $\pm$ 11.2	8.6 $\pm$ 8.5	12.9 $\pm$ 13.4
<b>Post VEGF (pg/mL), mean <math>\pm</math> SD</b>	11.8 $\pm$ 12.8	8.9 $\pm$ 8.9	14.8 $\pm$ 15.7

#### 4.4.6 Serum immune marker variability after exercise

The pre- and post-variability of serum IL-6 was significant for the group total ( $p = 0.003$ , Wilcoxon matched-pair signed-rank test) and for the intensive group ( $p = 0.01$ , Wilcoxon matched-pair signed-rank test) and tended to be significant in the moderate group ( $p = 0.09$ , Wilcoxon matched-pair signed-rank test) (Fig. 8).

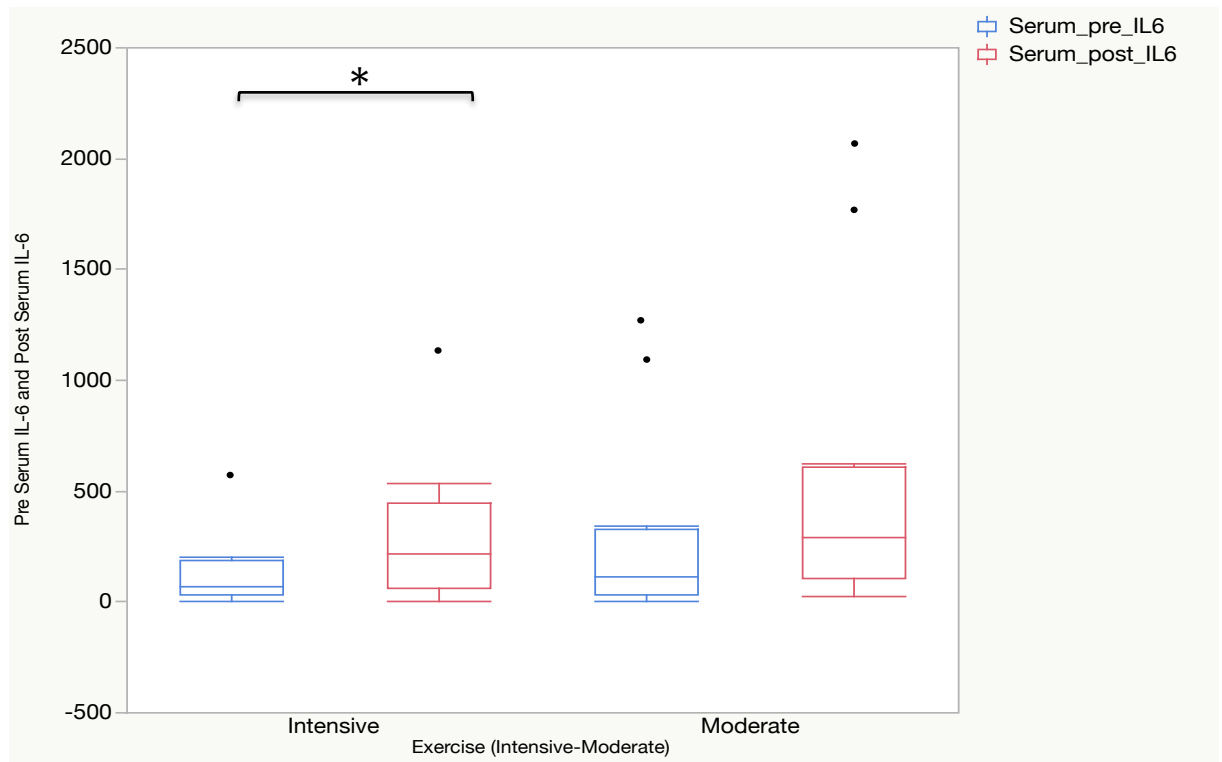


Figure 8. Box plot over serum IL-6 (fg/mL) pre- and post-exercise across groups ( $p < 0.05$ ).

The pre- and post-variability of serum IL-8 was significant for the group total ( $p = 0.01$ , Wilcoxon matched-pair signed-rank test) and for the moderate group ( $p = 0.02$ , Wilcoxon matched-pair signed-rank test) and not significant for the intensive group ( $p = 0.42$ , Wilcoxon matched-pair signed-rank test) (Fig. 9).

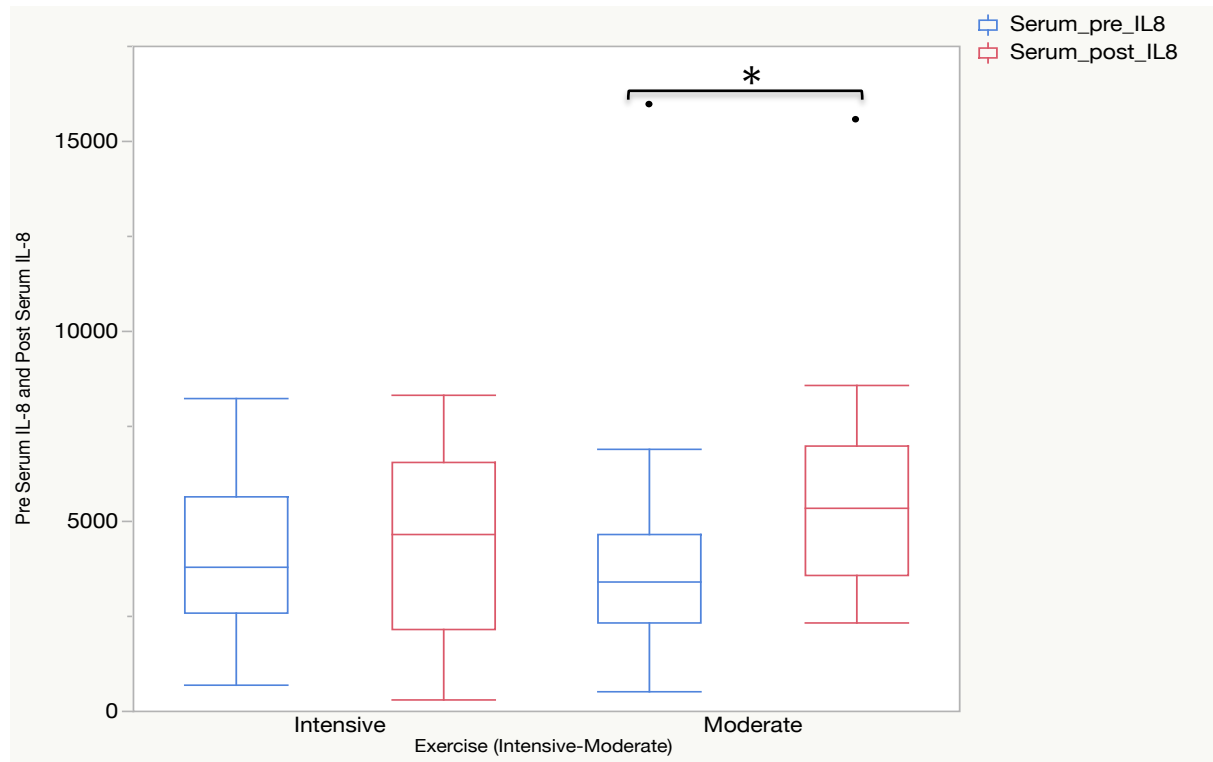


Figure 9. Box plot over serum IL-8 (fg/mL) pre- and post-exercise across groups. (\*  $p < 0.05$ ).

The pre- and post-variability of serum VEGF was not significant for the group total ( $p = 0.24$ , Wilcoxon matched-pair signed-rank test) or for the testing across groups, intensive or moderate ( $p = 0.73$ ,  $p = 0.27$ , Wilcoxon matched-pair signed-rank test).

#### 4.4.7 Correlation between serum and CSF levels

There was no significant correlation between serum and CSF levels of IL-6 and IL-8 in the baseline condition or after exercise ( $p$  values ranged between 0.37 and 0.99).

#### 4.4.8 Correlation between CSF levels of immune markers and mood ratings

PHQ-9 ratings were not significantly changed after exercise ( $p = 0.15$ ). At baseline, CSF IL-8 tended to be negatively correlated with the PHQ-9 score (Spearman's  $\rho = -0.49$ ,  $p = 0.06$ ) and, after exercise, CSF IL-8 was significantly negatively correlated with the PHQ-9 score (Spearman's  $\rho = -0.57$ ,  $p = 0.02$ ).

#### 4.4.9 Adverse events after lumbar puncture

Two participants withdrew from further participation after baseline sampling because of post-puncture headache. All headaches reported were clinically typical of post-puncture headache and were, in all cases, self-limited with spontaneous resolution after

a few days and did not hinder work or studies. No cases of severe complications occurred.

#### 4.4.10 Discussion

The rationale for the fourth study was based on the fact that biomarker studies often rely on matched healthy comparison subjects to determine plausible differences of interest between the clinical subjects and healthy participants. It is acknowledged that many confounders are not readily adjusted for, but the effect on common immune markers of interest in psychiatric research relating to the habitual exercise level and of PE are not fully known and are rarely studied especially regarding the intrathecal compartment.

We demonstrated that a physical activity intervention in healthy subjects results in alterations in the levels of certain cytokines in serum or CSF. A robust evidence base has confirmed the effect from PE on immune markers in the peripheral compartment; however, there is a paucity of studies that have addressed this issue in the intrathecal compartment. We found that, in paired samples from blood and CSF obtained from healthy participants, the degree of physiological variability in common immune markers can differ considerably and, for some cytokines, markedly as a result of physical exercise even at moderate intensity.

The effect that physical activity exerts on psychiatric disorders, mostly stress-related and depressive disorders, is believed to be the result of a contribution to improved stress resilience and a return to stress homeostasis (125,126). A recent clinical study on patients with fibromyalgia, a condition characterized by chronic inflammation, supported this concept. The patients had increased levels of serum IL-8 at baseline, as well as an increased stress axis and cytokine activity compared to healthy subjects. This aberration was reversed to the level of that of the healthy subjects at baseline by a single session of moderate physical activity while the healthy subjects, in line with our study, had an increase in the same parameters (127).

Further information emerging from this study was that many of the assayed analytes were below the detection limit. We believe, however, that this negative finding provides information that some cytokines are not heavily altered by physical exercise in healthy subjects and that they are generally low both at baseline and after exercise.

We have in the previous study (Study II) reported that CSF IL-8 levels were lower in suicide attempters than in healthy controls. In this study, CSF IL-8 levels were highest among participants with a higher habitual exercise level. Furthermore, CSF IL-8 levels were negatively correlated with mood ratings. However, the mood ratings were very low in the participants, and whether or not this reflects a mechanistic role for CSF IL-8 pertaining to mood merits further clarification.

Thus, the main finding in this study was that most immune markers in the CSF were generally low in healthy subjects, which has implications for the sensitivity of detection, but with no relevant variability as a result of physical activity. CSF IL-8 was significantly elevated, however, as a result of physical activity across both exercise

groups; i.e., from a more acute exercise challenge as well as from more moderate exercise over time. We also found that CSF IL-8 tended to be higher at baseline in the participants who had a higher level of habitual physical exercise. Furthermore, CSF IL-8 was negatively correlated with ratings of mood state among the participants both before and after exercise, suggesting that CSF IL-8 may be a state marker for mood and may be important for how exercise modulates mood. Serum IL-6 and IL-8 also display differential variability as a result of physical exercise, which is consistent with previous studies within this field. Collectively, this supports the notion that differences in the level of physical activity can affect biomarker levels in the intrathecal compartment and that efforts should be made to extend this type of analysis to a wider range of biomarkers.



## 5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

### 5.1 GENERAL DISCUSSION

This thesis has focused on identifying biomarkers relevant for predicting or providing new insights into plausible mechanisms underlying the biology that drives suicidal behavior. In this context, the hope is that findings from this and other researchers' work in this field may increase our understanding and in the future provide complementary tools for clinical assessment and new ways of targeting suicidal behavior with novel treatments that affects an aberrant immune activity.

Research in neuroscience is a challenge because of the immense complexity of the human brain, and, today, psychiatric research has abandoned hope of finding single mechanisms that explain the pathophysiology. This thesis has also been produced during a period of time when the hope that neuroinflammation may be a relevant construct for use in psychiatric disorder pathophysiology has proved to be hard to replicate for individual biomarkers, and the findings reported have not been consistent. In that context, the second part of the thesis has relevance in that it fills a knowledge gap regarding the effects of physical exercise in the intrathecal compartment, and by so doing, further broadens our insight into how exercise may be a relevant confounder when conducting clinical studies.

#### 5.1.1 Dysregulated immune system

The concept of chronic inflammation in this setting of studies is difficult to properly apprehend since there is no universal definition of such a state. There is, however, an abundance of studies that link increased morbidity and mortality with elevated levels of proinflammatory immune markers. A dysregulated or aberrant immune activity is perhaps a better description since inflammation, being in the classical sense, a reaction to pathogens or tissue damage, is not applicable in this context. The underlying causes of this dysregulation seem to converge on several underlying pathways, where an inherent genetic propensity for a strong immune response is one susceptibility factor, and depression may be the other side of the coin, bearing in mind the evolutionary advantage of a strong immune response for survival (128).

Furthermore, immune activation is strongly associated with the stress response system, and early adverse stressors, or repeated severe or chronic stressors, have a direct impact on immune activity, and epigenetic modifications can alter the genome to a state of increased proinflammatory immune activity (129,130).

##### *5.1.1.1 Evidence for inflammation in the pathophysiology of psychiatric disorders*

There are many lines of evidence for a role of the immune system in such psychiatric disorders as depression and suicidal behavior. There are several reports where increased levels of cytokines, CRP and chemokines are evident. Furthermore, epidemiological findings have provided interesting associations even prospectively when it comes to IL-

6 levels in childhood and a subsequent risk of depression (131). The same goes for evidence of prenatal or early childhood signs of inflammation or infection and the subsequent risk of mainly psychotic disorders (132,133). More convincing evidence comes from the important studies on IFN- $\alpha$  treatment and the risk of depression (134), as well as studies on lipopolysaccharide (LPS) injections with an evident increase in depressive symptoms in previously healthy subjects (135).

Furthermore, in the context of suicidal behavior, evidence for an aberrant immune activation as a plausible pathomechanism comes from observational studies, where the previously known observation of an increase in suicidal behavior during different seasons such as spring, seemed to be mediated by peaks in aeroallergens (136), and also that the use of intranasal steroids, which reduces the amount of airway-induced cytokines, was negatively associated to suicidal behavior (137). There are more interesting observational associations reported, that the percentage of seropositivity for the common neurotropic parasite *Toxoplasma gondii*, that knowingly induces an immune response, was significantly associated to a risk of suicidal behavior. An association that could be viewed upon, as corroborative for an underlying immune dysregulation, being involved in the unadaptive behavioral response involving suicidal behavior (136).

#### *5.1.1.2 Innate immune dysregulation*

Based on numerous studies, both preclinical and clinical, which argue that increased proinflammatory cytokines and chemokines have a role in the pathophysiology of depression, a role for a dysregulated activity mainly in the innate immune system is proposed. Innate immune mechanisms are the first line of defense against internal and external pathogens or stressors that challenge homeostasis or cell function. The interesting link between depression and other diseases linked to an aberrant immune activity, such as diabetes type 2 and obesity, corroborates this idea since these disorders are also suggested to be the result of a dysregulation in the innate immune system (138).

Furthermore, even psychological stress has the ability to activate components of the innate immune system, and long-term stress or severe adverse events have the capability to alter these markers together with an alteration of the stress response system (139,140). Microglia within the brain can be activated and express the different aberrant cytokines and chemokines in depression and, as opposed to peripheral effects of cortisol, microglia expansion and cytokine expression is increased intrathecally by cortisol (140). Signs of microgliosis is also evident in suicidal behavior according to post mortem studies (140).

#### **5.1.2 Peripheral compartment vs. the intrathecal compartment**

It seems to be rational to assume that what goes on in the intrathecal compartment is a better measure of the immune activation signature pertaining to the brain and its surrounding structure, rather than the peripheral compartment. There are, however, obvious issues encountered in conducting CSF studies, since they involve a much more invasive procedure with known complications, such as post-spinal headache. From our

studies, and other CSF studies on suicidal behavior, it is evident that the peripheral compartment does not mirror the intrathecal compartment, and with no signs of correlation, indicating that these compartments are segregated in terms of immune marker expression, as was also the case in a previous exercise study, as well as results from our own exercise study reported in this thesis (63,141,142).

This issue is also discussed in studies conducted on known neuroinflammatory disorders such as MS (143). The brain was previously regarded as an immune privileged organ; however, this view has been discarded for a long time and the brain is now referred to as being immune specialized (144). There is a constant surveillance by peripheral immune cells, but also from points of entry through the different barriers surrounding the brain, where not only the generally known blood-brain barrier (BBB), but also barriers between the blood and meninges and the choroid plexus are regions where cross-talk between immune cells takes place (144). An intriguing discovery in 2015 also revealed that the brain is not only not as immune privileged as it was once thought to be, but that the brain also has a lymphatic drainage, which further opens up new ideas about how a neuroinflammation is instigated and may become chronic (145).

### 5.1.3 A role for VEGF in attempted suicide

There are quite a few studies, mainly preclinical, suggesting the putative role of VEGF in certain neuropsychiatric disorders, mainly depression. In chronic-stress paradigms in rodents VEGF expression in the hippocampus is down-regulated as well as its target receptor (146). There are also preclinical studies that have demonstrated VEGF down-regulation in response to treatment with glucocorticoids (147,148). Exercise paradigms and, enriched environment paradigms have shown an increase in the expression of VEGF, with direct importance in, e.g., hippocampal neurogenesis (81,149). There are interesting links from animal studies that provide evidence that VEGF is a mediator involved in how antidepressants such as SRIs, PE, and electroconvulsive seizure (ECS), exerts their therapeutic effect (150–155).

Clinical studies in humans have also provided corroborative evidence for VEGF to have a relevant role mainly regarding depression or stress-related disorders (73). However, due to the general complexity of clinical studies, the results are rather conflicting. One study reported that high levels of VEGF mRNA in peripheral leukocytes showed a significant correlation with the depression level (156). Furthermore Åsberg and collaborators reported that elevated plasma levels of VEGF were associated with prolonged sick-leave in women with a stress-related disorder, while another Japanese study reported decreased levels corresponding to stressful military training (157,158).

Most clinical studies have reported elevated peripheral VEGF levels that correspond with psychiatric disorders (159–161). VEGF is a component of the innate immune response, and the main regulator for VEGF is hypoxia via the promoter hypoxia inducible factor 1 (HIF-1). Logically, the effect from external or internal stressors is an increase in VEGF as a reparative response. Recent reports have studied known polymorphisms for both *VEGF* and the *VEGFR-2* (162–164) where polymorphisms associated to higher expression levels both regarding peripheral VEGF levels and

VEGFR-2 levels were associated to recurrent depression, and, in one study, by Viikki and collaborators to treatment resistant depression, but not to treatment response in the same study (162). Overall, these results, do not align in an intuitive way with the neurotrophic hypothesis of depression, where levels would rather be expected to be low.

Contextualizing our studies with more recent reports have lifted the idea that low, and plausibly a hyporesponsive VEGF function may be a biomarker for treatment resistance in depression or more severe depressive states even characterized by risk of suicidal behavior (79,165). Studies I and II within this thesis present a key finding that VEGF levels are low both in plasma and in the CSF, and that low plasma levels were predictive of completed suicide (141,166). Studies and reports by Halaris and collaborators have discussed that elevated peripheral levels may serve as a biomarker for depression suggesting that elevated levels are a physiological response to stimulate neurogenesis, while, speculatively, low baseline levels could imply that the physiological ability to self-heal is impaired and therefore results in treatment resistance (79,167). A recent report presented evidence in line with this idea, where an optimal cut-off level for plasma VEGF could predict treatment response with antidepressant treatment in MDD subjects and where low baseline levels predicted non-response (165). Clinical studies using antidepressant pharmacological therapies have failed to show associations between treatment response and concomitant increases in VEGF. However in treatment resistant depressive subjects electroconvulsive treatment (ECT) was associated to an increase in serum VEGF levels, and this was also associated to MADRS decline after intervention (168). Furthermore, a study, using sleep deprivation as intervention could report increases in VEGF levels and a correlation to improvement in mood in depressed subjects, while BDNF levels, remained unaltered (169). Taken together, these reports could indicate that VEGF response is to a larger degree dependent of non-pharmacological treatment options in humans as compared to regular pharmacological treatment options.

While there are robust evidence from preclinical studies on neurogenesis being a relevant mechanism of action, a recent report proposes new interesting mechanisms regarding how VEGF may be efficacious in treatment and in which VEGFR-2, in an animal model was evidently involved in synaptic plasticity in the hippocampus, acting by, modulating the post synaptic responses by the N-methyl-D-aspartate type of glutamate receptors (GluNRs), thereby being of direct importance in modulating fear-learning and memory consolidation.

From the above perspective including studies presented after our initial clinical studies on the role of VEGF in suicidal behavior, we believe that the finding from Study I in which low levels of plasma VEGF were a significant predictor of completed suicide in a cohort of suicide attempters, aligns our results with the concept of treatment resistance if completed suicide is to be regarded as a more severe phenotype and indicator of treatment resistance. Furthermore, Study II, assessing CSF levels of VEGF, corroborates in a way, this idea since CSF levels were significantly lower in suicide attempters compared to healthy subjects. However, a relevant psychiatric comparison group such as MDD subjects without suicidal behavior would have further strengthened this finding.

A model for VEGF in treatment-resistant depression (TRD) and suicidal behavior is presented below (Fig. 10).

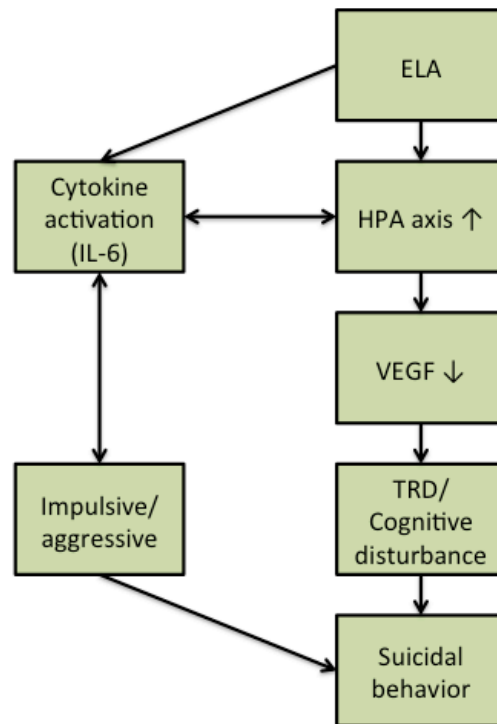


Figure 10. A multifactorial model for VEGF down-regulation as a mediator and biomarker in the chain leading to treatment resistance and in some instances suicidal behavior, including risk for completed suicide.

#### 5.1.4 Physical exercise, immune modulation and therapeutics

In the Introduction, the profound effects of PE are described in more detail. PE affects the immune system by mobilization of white blood cells and long-term effects on cytokines. The mechanisms for these alterations are thought to be achieved acutely via activation of the HPA axis, as well as the SNS via its effector molecules, as well as via signaling molecules derived directly from the skeletal musculature (myokines) (170).

There is evidence from several epidemiological studies that a sedentary lifestyle is associated with an increased risk of depressive symptoms, and physical exercise is associated with a reduced risk of depressive symptoms in a dose-response fashion (171,172). The differential effects in human brains of physical exercise are known from several clinical studies. Exercise has effects on cognitive domains, mainly visuospatial, thereby reducing cognitive decline in elderly populations, and imaging studies have also shown increased volumes in several brain regions, mainly the hippocampus, thereby providing indirect evidence that neuroplasticity occurs (125,173).

There is evidence from clinical, as well as rodent, -studies that exercise modulates the monoaminergic system and has effects on NE, dopamine, and serotonin turnover (173).

Rodent studies also provide evidence that physical activity increases several neurotrophic growth factors, such as VEGF, BDNF, FGF-2, and insulin-like growth factor (IGF)-1; furthermore in humans, there is evidence for a transient peripheral increase of BDNF from PE (173,174) and even evidence for a central increase in BDNF (175). In our exercise study, we could not report any significant changes regarding VEGF in serum, and also VEGF assayed in the CSF was below the detection limit. This could be argued to be inconsistent with the Discussion above, however, at least from unpublished data from our lab-group VEGF levels generally are low in the intrathecal compartment and commonly falls close to or below the detection limit.

Since the hippocampus is considered to be of importance in the pathophysiology of depression, as well as suicidal behavior (72,176,177), the connection with exercise-induced neurogenesis in the hippocampus region, as well as reversing stress-induced depression from animal studies, provides support that physical exercise may be considered to be therapeutic and to act preventively through increased stress resilience (81,125,154,173).

A rather new study from a research group at Karolinska Institutet also provide new interesting insights into how physical activity may prevent and increase stress-resilience via induction of the muscle enzyme PGC-1 $\alpha$ 1. The PGC-1 $\alpha$ 1 enzymatic pathway increases the production of kynurenine aminotransferases (KATs) that catabolize kynurenine into kynurenic acid, a metabolite that is unable to cross the BBB. The study provides evidence from both transgenic mouse models and a clinical component that these effects actually occur. This opens up an interesting link in the connection between the before hand discussed cytokine aberration and a proinflammatory milieu as seen in chronic stress conditions. The induction of the kynurenine pathway previously discussed as an effect of increased inflammation and the role of PE as a possible means of preventing depression via increased resilience through the induction of a beneficial enzymatic pathway and, furthermore, as a way of treating depression via this mechanistic pathway (178).

In this context, our Study IV also provides evidence that PE has a profound effect on immune markers, not only peripherally, but also in the intrathecal compartment. Our findings on CSF interleukin alterations are interesting, since, also for CSF IL-8, they seem to be correlated with ratings of mood. An interesting observation, in the light of the report showing that low CSF IL-8 levels were corresponding to anxiety in suicide attempters (123), and where PE could be an important tool in modulating an aberrant mood state, provided that our results indicate a plausible mechanism for how this may be achieved.

Furthermore, on a more general level, in this type of research, there are discussions about whether patients with an aberrant immune activity are the ones that benefit the most from anti-inflammatory treatment and PE. Corroborative evidence in line with this idea is presented in the study on TNF- $\alpha$  inhibitor treatment for patients with treatment-resistant depression by Raison and collaborators, where CRP levels at baseline predicted response (179), and also an exercise study on patients with depression where baseline levels of TNF- $\alpha$  were a predictor of treatment response (180).

A schematic model is presented below (Fig. 11) describing possible mechanisms resulting from PE in both the peripheral compartment and the intrathecal compartment with effects on different modulators and pathways implied to be of importance in depression and suicidal behavior.

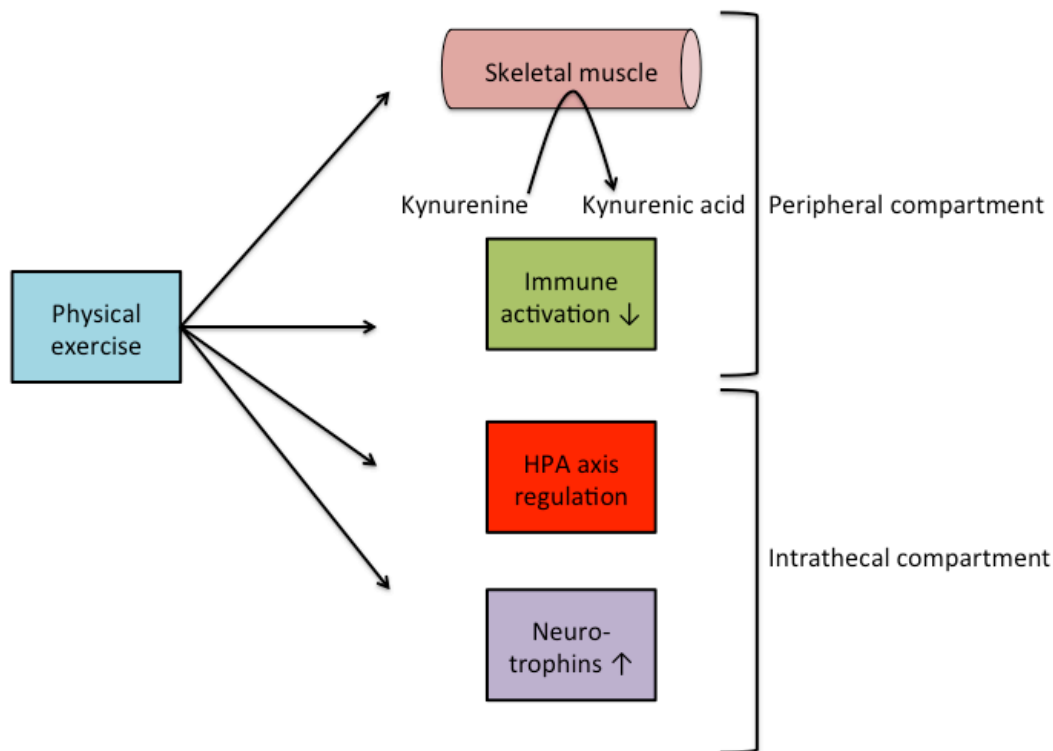


Figure 11. A schematic model of beneficial effects of PE, i.e., improvement in stress resilience and return to homeostasis.

### 5.1.5 Conclusion

The studies reported in this thesis have introduced new data regarding potential biomarkers when assessing suicide risk and put forward ideas about how one can assess or understand inconsistencies between studies by aligning one of the included study's design with the concept of endophenotypes, which is as a mean of deconstructing a broader clinical phenotype. Treatment resistance seems to be of utter importance, as reported by a Finnish longitudinal clinical study, with the interesting results indicating that the time spent in depression seemed to be the most important predictor of future suicide attempts (181) and, emphasizing the importance of having remission as a primary goal when treating patients suffering from MDD and adding support that suicide attempts are a more severe phenotype in MDD and an indicator of treatment resistance. Furthermore, assessing personality traits, or cognitive measures of impulsivity would seem to be important since studies from our group have provided data that personality domains have a further impact on immune markers even in a cohort of patients presenting with suicide attempts. We also stress that physical exercise has an impact on immune markers even in the intrathecal compartment, and these alterations could provide new ideas about how physical exercise may produce its

therapeutic effect, and that, in some settings, physical exercise could be a relevant confounder when conducting biomarker studies.

#### *5.1.5.1 Studies I-III*

From the first follow-up study (**Study I**), we report that low plasma levels of VEGF may be of predictive value regarding the risk of completed suicide in suicide attempters and, in that context, also a marker of treatment resistance. This must be replicated, however, in independent samples.

From our second study (**Study II**), we report cross-sectional data indicating that low CSF levels of VEGF and IL-8 may constitute a biomarker for suicide risk as compared to healthy controls.

In the third study (**Study III**), based on cross-sectional data, we also report that IL-6 levels in plasma and in the CSF may be confounded by personality traits and the method of attempted suicide (violent/non-violent). We found a correlation mainly regarding IL-6 plasma levels and impulsiveness and sensation seeking traits, and to a lesser extent correlations between IL-6 CSF levels and sensation seeking traits. We believe that this merits further caution when assessing cytokines as biomarkers in suicide research, although today study designs often include neurocognitive testing. IL-6 may also be associated with the choice of method, but this result did not reach statistical significance in our study. The study corroborates the idea of endophenotypes as relevant constructs to consider, and motivates the use, of dimensional phenotypic traits, conceptualized in the RDoC, when attempting and designing studies concerning suicidal behavior.

#### *5.1.5.2 Study IV*

The final study (**Study IV**) included in this thesis was organically derived to address potential confounding concerning the level of physical activity when comparing patients and healthy controls. This has not been extensively studied especially regarding the intrathecal compartment. Longitudinal paired data from both plasma and the CSF, as used in our study, is unique in this context, to our knowledge, and this experimental clinical study hopefully provides important information, not only in the field of psychoneuroimmunology, but also in the field of studies on other neurological inflammatory disorders. The results call for caution when attempting biomarker research in general, since the level of habitual physical activity, or any recent physical exertion, might have profound effects and this should therefore be either controlled for or ensured that conditions are equal with regard to physical activity. The results are also of interest when defining the plausible effect of immune markers as being therapeutic regarding neuropsychiatric disorders since we could also report that CSF IL-8 was correlated with mood, indicating a plausible role as a state marker. Furthermore, our paired sampling implies that the immune marker expression is segregated between the peripheral and the intrathecal compartment.

#### *5.1.5.3 Summary*

In summary, this thesis titled “Neuroinflammatory Biomarkers in Suicidal Behavior” has provided new information, mostly regarding VEGF as a potential biomarker of interest in suicidal behavior. Furthermore, the role of CSF IL-8 as a biomarker for both



suicidal behavior and mood is suggested by our results. We also provided new relevant input from screened healthy subjects on the effects of physical exercise and the habitual exercise level regarding immunological biomarkers, thereby reporting on the high degree of variability for some cytokines, also within the intrathecal compartment.

### 5.1.6 Strengths

#### 5.1.6.1 *Studies I-III*

The strength of the first part of the thesis was the thoroughly clinically assessed cohort of suicide attempters, with a strict protocol regarding biological sampling. Another was that we could include follow-up data on the cause of death. Paired sampling was provided from both plasma and CSF. The study sample was moderately large in this context. Moreover, the samples had never thawed prior to our analysis.

#### 5.1.6.2 *Study IV*

The strengths regarding the second part of the thesis was the careful inclusion procedure which assured that the participants were completely healthy and without any history of psychiatric or medical problems. They were also assessed regarding concomitant immune activation using routine laboratory screening, and they were urged not to take any medication, even painkillers, during the study period. Furthermore, all participants were nonsmokers and there was no self-reported substance misuse. The paired sampling at both baseline and after exercise according to a strict protocol was unique in this context of studies.

### 5.1.7 Limitations

#### 5.1.7.1 *Studies I-III*

An important limitation regarding the first part of the thesis is the cross-sectional biological sampling and, regarding Studies II and III, the overall cross-sectional design that excludes the possibility of determining causality. The type of assay methodology is also an issue since reports have raised concerns about the reliability between the type of methodology and the producer of the assay technology, which may raise issues in the replication of study results (70). Another issue regarding the first part of the thesis is the long duration of time between sampling and assay, which has also raised concerns about the stability of immune markers, which may vary for each marker, even though they have never been thawed previously (184,185).

Data on the subjects' smoking habits were not assessed in a structured manner and hence were not adjusted for, but regarding peripheral VEGF levels there is one report stating that cigarette smoking did not affect VEGF levels in subjects with depression (186). We did not have data on socioeconomic status that also could be a relevant confounder in relation to cytokine levels. As for the healthy controls, these were all men and they were not age-matched. Patients not willing to undergo lumbar puncture or not willing to undergo any biological sampling at all could also affect the generalizability of the results in the first part of the thesis since these patients may have intrinsic differences in, e.g., personality traits or other phenotypical characteristics. Furthermore, the patient cohort could be determined to be a high-risk cohort, with a high degree of comorbidity and a high percentage of completed suicide attempts at

follow-up. This also raises issues concerning the generalizability of the findings since this group does not necessarily reflect the larger group of patients with issues regarding suicidal behavior.

#### 5.1.7.2 *Study IV*

Limitations need to be pointed out regarding the second part of this study. The lack of supervision regarding the exercise protocol may raise concerns regarding protocol compliance. Differences in CSF levels are difficult to fully interpret and might also be a consequence of close-in-time physical exertion in a mere mechanistic way, e.g., extravasation of immune markers or changes in gradients, rather than a true increase in the expression of the immune markers that were assayed. The relevance of mood alterations and its correlation with one immune marker should be interpreted with caution since this reporting was incomplete, and the overall ratings in mood were low, as well as the alterations in mood rating after exercise being only subtly changed.

## 5.2 FUTURE DIRECTIONS

Psychiatric research is on the rise. There is an ongoing technological improvement within the field of genetics and collaborative efforts are key in increasing sample sizes as well as the generalizability of findings.

Clinical research is complex and requires careful ethical consideration to make certain that, for individuals exposed to research, which may involve extra tribulations, all measures have been exhausted to ensure that the study design is well thought out. There are no animal models for suicidal behavior, which is limited to humans. However, there are known risk factors for which there are animal models, such as depressive-like behavior.

Future studies should include imaging, genetic and epigenetic determination, neurocognitive domain testing together with the biological sampling, to investigate the biochemical milieu within the patient, also having in mind assessment of the stress hormone axis level of activity. Sampling needs to be performed at multiple time periods and ways to assess immune markers longitudinally must be found. One must also bear in mind for the study, the use of relevant comparison subjects, both psychiatric and healthy participants, with relevant matching based on different potential confounds. This kind of broad assessment is already being performed in other fields of psychiatric research, and with an increasing amount of optimism. Hopefully, these studies will also include the phenotypical characteristic that manifests itself through suicidal behavior.

Furthermore, a few studies have been conducted on different types of anti-inflammatory treatments for affective disorders, and the use of substances that modulate the glutamatergic system and the NMDA receptor is also an encouraging twist on how to move on from our current understanding of immune dysregulation in neuropsychiatric disorders. Even more new studies using physical activity, as a treatment should also attempt to determine what mechanisms are important, using results such as ours to understand what truly happens in humans after physical exercise.

We have the further ambition within our own group to use the collected data on personality and the biological samples from the exercise study to broaden our analysis regarding this cohort in order to explore other potential pathways that may be altered as a consequence of PE, as well as plausible connections to personality traits, with the aim of finding relevance regarding neuroinflammatory and psychiatric disorders. Furthermore, assaying BDNF in our subjects would be interesting in the light of previous research.

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